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**Design and development of a novel functional pasta:
microbiological characteristics and
impact on gut microbiota**

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Riassunto

Il ruolo dell'alimentazione nel contribuire allo stato di salute sta ricevendo una sempre maggiore attenzione. Inoltre, le proprietà degli alimenti possono essere migliorate con l'aggiunta di ingredienti funzionali, tra cui probiotici e prebiotici, in grado di agire sul microbiota intestinale.

L'obiettivo di questa ricerca è stato quello di sviluppare una nuova pasta funzionale integrale, contenente un microrganismo probiotico sporigeno, il ceppo *Bacillus coagulans* GBI-30, 6086 (BC³⁰) e β -glucani d'orzo, e di valutare le sue proprietà microbiologiche, e gli effetti del suo consumo sul microbiota intestinale, in due contesti, il diabete di tipo 1 (T1D) e l'obesità.

In fase di messa a punto del prodotto funzionale, è stata innanzitutto valutata la stabilità del ceppo probiotico, utilizzando metodi microbiologici classici e analisi di qPCR. I risultati hanno mostrato come il ceppo risulti stabile nei processi di produzione e cottura della pasta, e sia in grado di sopravvivere al transito nel tratto gastrointestinale.

Nell'ambito del diabete, è stato effettuato, in primo luogo, uno studio caso-controllo, volto ad indagare eventuali differenze del microbiota intestinale di bambini sani e bambini a rischio di sviluppare T1D. Le analisi di PCR-DGGE hanno mostrato come alcune specie risultino caratteristiche della condizione di pre-diabete. In un secondo studio, gli effetti dell'amministrazione della pasta funzionale e dei suoi ingredienti (in termini di modulazione della composizione microbica e produzione di acidi grassi a catena corta) sono stati valutati in un modello *in vitro* del colon, TIM-2, sul microbiota fecale di bambini con diversa suscettibilità al T1D. Sono state individuate specifiche relazioni dieta-effetto sul microbiota, anche ampliando le informazioni disponibili rispetto al ceppo probiotico e ai β -glucani.

Infine, è stato valutato l'impatto del consumo della pasta funzionale in uno studio clinico che ha coinvolto soggetti sovrappeso/obesi. Le analisi di PCR-DGGE e qPCR hanno mostrato come il nuovo prodotto non abbia modificato in modo sostanziale la composizione microbica intestinale, e non abbia portato ad aumenti di specifici gruppi microbici correlati ai diversi ingredienti della pasta funzionale nella totalità dei soggetti, a causa della marcata variabilità individuale. Stratificando la popolazione in sottogruppi più omogenei, e correlando i dati microbiologici e clinici, sono comunque emersi alcuni risultati interessanti relativi al nuovo prodotto.

In conclusione, questo lavoro ha mostrato come la nuova pasta funzionale costituisca un prodotto di alta qualità per le sue proprietà nutritive, e per gli effetti positivi osservati sia *in vitro* a livello del microbiota intestinale, sia dai risultati clinici e microbiologici. Ulteriori studi saranno utili per approfondire ed ampliare le conoscenze acquisite.

Abstract

Foods are not intended anymore only as sources of nutrients and energy, but also as factors that can influence health status. In this perspective, food properties can be improved by the addition of functional ingredients, e.g. probiotics and prebiotics, that can positively act on gut microbiota. Thus, the aim of the present research was the development of a novel whole grain functional pasta, added with the spore-forming probiotic *Bacillus coagulans* GBI-30, 6086 (BC³⁰) and β -glucans from barley, and the investigation of its microbiological properties and effects on gut microbiota, in two contests, type 1 diabetes (T1D) and obesity condition.

In particular, the stability of the probiotic strain was investigated during the development of the functional product, through conventional plating and qPCR analyses, providing evidences related to its viability following pasta-manufacturing and cooking processes, as well as to its survival to the transit through the gastrointestinal tract.

In type 1 diabetes contest, a case-control study was firstly performed for assessing differences in gut microbiota composition between children at risk for T1D and healthy children. PCR-DGGE analyses showed that certain species were characteristics of the pre-diabetes condition. Then, the effects (i.e. modulation of microbiota composition and short-chain fatty acid production) of the administration of the functional pasta and its ingredients were investigated with respect to fecal microbiota from children with different susceptibility to T1D, using the TIM-2 model of the proximal colon. Specific diet-microbiota relationships were individuated, also expanding available information about the probiotic and prebiotic ingredients.

Finally, the impact of the consumption of the functional pasta was investigated in a clinical trial involving overweight/obese subjects, through PCR-DGGE analyses and qPCR. The novel product did not substantially affect gut microbiota composition, and, in the total group of subjects, it did not caused increases of specific microbial groups reported to be modulated by the different functional pasta ingredients, due to the high inter-individual variability. Stratifying the population in smaller, more homogeneous groups, and correlating microbiological and clinical data allowed to unveil some interesting findings related to the new product.

In conclusion, the present research indicated that the novel developed pasta constitutes a high-quality product for its nutritional properties, and for positive effects observed both *in vitro* at the gut microbiota level, and from clinical and microbiological results. However, further studies will be helpful to deepen achieved knowledge on this new product.

1. Introduction

The concept of functional food has emerged as a result of extensive research on the relationship between diet and health. Even if a unified definition has not yet been established (Bigliardi and Galati, 2013), the idea is that “a functional food consists in, or appears similar to, a conventional food. It is part of a standard diet and is consumed on a regular basis, in normal quantities. It has proven health benefits that reduce the risk of specific chronic diseases or beneficially affect target functions beyond its basic nutritional functions” (Doyond and Lebreque, 2008). Thus, a certain functional food can be introduced with the diet, e.g. in substitution to a conventional food, representing a successful strategy for the introduction of component with beneficial effects on the health. Probiotics and prebiotics constitute functional ingredients able to act on gut microbiota, and can be used in the development of functional foods.

In the following sections general aspects relative to probiotics and prebiotics, with a specific focus on the strain *Bacillus coagulans* GBI-30, 6086 and β -glucans, and the development of new functional products will be presented. In addition, a short overview on gut microbiota, its involvement in diseases, and its modulation through the diet will be provided.

Probiotics

According to the currently adopted definition, probiotics are defined as “live microorganisms that, when administrated in adequate amounts, confer a health benefit on the host” (FAO/WHO, 2002; Hill *et al.*, 2014). This implies that a probiotic microorganism should fulfil different criteria e.g. being present in adequate number of viable cells in a product, being able to survive the transit through the gastrointestinal tract, and providing a specific health benefit to the host (Collado *et al.*, 2009). The mechanisms through which probiotics exert their effects can include competitive exclusion of pathogens due to production of antimicrobial compounds and inhibitory agents, e.g. bacteriocins, and competition for nutrients and adhesion sites, enhancement of the intestinal barrier function, pH modification, modulation of inflammatory responses through interaction with immune cells in the gut (Collado *et al.*, 2009; Papadimitriou *et al.*, 2015). Probiotics have been applied in many contests, resulting active in the treatment of intestinal diseases, for example intestinal bowel disease (IBD), infectious diarrhoea, antibiotic-associated diarrhoea, Crohn’s disease, and also at a systemic level, e.g. allergic diseases (Collado *et al.*, 2009; Iannitti and Palmieri *et al.*, 2010).

However, these mechanism and effects can not being generalised. In fact, probiotic properties represent strain-specific features, that means that effects have to be investigated case by case (Power *et al.*, 2014). At the same, this indicates that taxonomic identification is essential to connect a strain to a specific effect on health and to safety issues. From the safety point of view, at the European level, a probiotic strain must be included among the species of the QPS (Qualified Presumption of Safety) list, drafted by the European Food Safety Authority (EFSA) (EFSA, 2007).

Traditionally, most of bacterial probiotic strains belong to the genera *Lactobacillus* and *Bifidobacterium*, but strains belonging to other genera e.g. *Enterococcus*, *Lactococcus*, *Leuconostoc*, *Pediococcus* and *Bacillus* are also available. In particular, *Bacillus* strains have been used as probiotics for at least 60 years, considering that the Italian product Enterogermina, a *Bacillus clausii* preparation, was registered in 1958 as an OTC medicinal supplement (Cutting, 2011). The genus *Bacillus* present the specific characteristic of forming spores, very resistant structures able to withstand adverse environmental conditions, e.g. extremes of heat, UV radiation, exposure to solvents, enzymes. Spores are dehydrated and, when exposed to appropriate nutrients, will germinate, in a process requiring just few minutes, in which water enters the spores, breaks the spore coats, making vegetative cell able to resume and grow (Cutting, 2011). Thanks to their spore-forming nature, probiotics belonging to the genus *Bacillus* present improved characteristics of resistance with respect to other non spore-former probiotics; in fact, they are able to resist to high temperatures, they can be stored at room temperature in a desiccated form without any deleterious effect on viability, and they can resist to low pH of the gastric barrier (Cutting, 2011).

The strain Bacillus coagulans GBI-30, 6086 (BC³⁰)

Bacillus coagulans was first isolated from coagulated milk by Hammer in 1915 (Hammer, 1915), later described in 1933 by Horowitz-Wlassowa and Nowotelnova as *Lactobacillus sporogenes*, and subsequently reclassified as *B. coagulans*. From a taxonomic point of view, this species belong to phylum Firmicutes, and to the family of Bacillaceae. Strains belonging to this species are Gram-positive bacteria, spore-forming, rod-shaped, facultative anaerobes, and they produce lactic acid from fermentation of maltose, mannitol, raffinose, sucrose and trehalose (De Vecchi and Drago, 2006). The species *B. coagulans* is considered a safe species, since it is included in the QPS list edited by EFSA (EFSA, 2007), updated to the 4th revision in 2016.

Among strains belonging to this species, the strain *B. coagulans* GBI-30, 6086 (BC³⁰) is one of the most studied and characterised one. *Bacillus coagulans* BC³⁰ is a commercial, patented probiotic strain, produced by the company Ganeden Biotech, and it received GRAS (Generally Recognized As Safe) status from U.S. FDA in 2012, following presentation of toxicological studies (Endres *et al.*, 2009; Endres *et al.*, 2011). Moreover, the genome of the strain has been recently sequenced (Orrù *et al.*, 2014), and integrated phenotypic and genomic analyses have confirmed the safety of the strain (Salveti *et al.*, 2016).

This probiotic microorganism is available as dietary supplement, and moreover, it has already been included in a broad range of food products and beverages available in U.S. market.

The effects of this probiotic strain on the host health have been investigated in several *in vitro*, in animal model and *in humana* studies. This strain has been reported to have beneficial effects at different levels. Regarding to the gastrointestinal (GI) compartment, *B.coagulans* BC³⁰ resulted effective in the treatment of symptoms related to gastrointestinal disorders, such as IBS and intestinal gas (Dolin, 2009; Hun, 2009; Kalman *et al.*, 2009), and it could act as a coadjuvant in the response towards pathogens in colitis (Fitzpatrick *et al.*, 2012). It can act at a systemic level against specific autoimmune diseases, such as rheumatoid arthritis, alleviating the symptoms, positively acting on inflammatory markers, and improving the overall health and motor skills of patients (Mandel *et al.*, 2010), and it has been shown to enhance the immunological response in common viral infections of the respiratory tract (Kimmel *et al.*, 2010). *Bacillus coagulans* BC³⁰ has also been recently reported to reduce exercise-induced muscle damage, improving recovery, and maintaining physical performances subsequent to damaging exercise (Jäger *et al.*, 2016).

Up to now, few studies investigated the effects of the probiotic strain on the modulation of the intestinal microbiota. In the *in vitro* study of Honda and colleagues (2011) the probiotic strain did not influence the components of microbiota, while in following studies modulations of some microbial groups were showed. In particular, *B. coagulans* BC³⁰ showed an effect in increasing the levels of *Clostridium lituseburense*, *Eubacterium rectale* and *Faecalibacterium praunsnitzii*, when administered together with a prebiotic, i.e. FOS, in an *in vitro* study with microbiota from healthy older people (Nyangale *et al.*, 2014). Moreover, in a clinical trial involving healthy elderly individuals, the administration of the probiotic strain significantly increased the levels of *F. praunsnitzii* (Nyangale *et al.*, 2015).

Prebiotics

Prebiotics are substrates that selectively promote the proliferation and/or activity of microbial bacteria that reside in the colon. They are defined as “selectively fermented ingredients that allow specific changes, both in the composition and/or activity in the GI microbiota, that confer benefits upon host well-being and health” (Gibson *et al.*, 2010). In order to be classified as a prebiotic, a food component must be neither hydrolysed by the host enzymes nor absorbed in the upper part of the GI tract, and it must be a selective substrate for one or a limited number of beneficial bacteria in the colon (Roberfroid *et al.*, 2010).

Most commonly employed prebiotics belong to the category of dietary fibre, in particular soluble fibre that includes inulin, fructooligosaccharides (FOS), and galactooligosaccharides (GOS), xylooligosaccharides, β -glucans, resistant starch (Gibson *et al.*, 2010).

The effects of prebiotics are attributed to the stimulation of beneficial bacteria, historically correlated to the genus *Bifidobacterium* and *Lactobacillus* (Cummings *et al.*, 2001), but also species such as *Ruminococcus bromii*, *Roseburia intestinalis*, *Eubacterium rectale* and *Faecalibacterium prausnitzii*, belonging to cluster IV and XIV of clostridia, should be considered as emerging candidates (Conlon and Bird, 2015). In fact, since these microorganisms can influence the production of short-chain fatty acids (SCFA), they represent important contributors to host health (Conlon and Bird, 2015).

The role of prebiotics in promoting health and reducing risk of gut and systemic diseases have been deeply reviewed (Gibson *et al.*, 2010; Clark *et al.*, 2012) and consistent evidences related to the improvement of bowel and immune function, and metabolic health in humans have been found (Conlon and Bird, 2015). Even if only a limited number of clinical trials have explored the effect of prebiotics on microbiota composition and metabolic markers simultaneously, these studies demonstrated the modulation of gut microbiota and the parallel improvement of insulin sensitivity, low-grade chronic inflammation, and lipid metabolism (Brahe *et al.*, 2016).

β -glucans

The β -glucans are a group of non-starch polysaccharides, that represent the soluble fibre fraction contained in the aleurone and subaleurone layer of different cereals and, in particular, barley and oat (Arena *et al.*, 2014). Different studies investigating the prebiotic potential of β -glucans *in vitro* and *in humana* are available. A functional pasta enriched in β -glucans from barley showed positive effect *in vitro* on the proliferation of four probiotic strains belonging to the genus *Lactobacillus* (Arena *et al.*, 2014). An increase of *Lactobacillus* spp., together with an increase

in *Bifidobacterium* spp. was reported in an *in vitro* study in which β -glucans from barley were inoculated with fecal microbiota (Hughes *et al.*, 2008). Moreover, Zhao and Cheung (2011) indicated how β -glucans from different sources, such as seaweed, barley, mushrooms, showed a bifidogenic effect comparable with that obtained by using inulin (Zhao and Cheung, 2011). Finally, two clinical trials dealing with β -glucans consumption reported a significant increase in the level of *Bifidobacterium* spp. (Mårtensson *et al.*, 2005; Mitsou *et al.*, 2010), that was more pronounced in older subjects (Mitsou *et al.*, 2010). These researches confirmed the bifidogenic effect of barley β -glucan previously shown *in vitro*. In a third study, no modulation related to the genus *Bifidobacterium* were observed after the consumption of a pasta containing β -glucans from barley, while clostridial species, e.g. *Roseburia hominis* and *Ruminococcus* spp. increased (De Angelis *et al.*, 2015).

At a systemic level, evidences from literature highlight the positive effects of β -glucans on conditions related to the metabolic syndrome, i.e. obesity, hypertension, hyperglycemia, and dyslipidemia (Cloetens *et al.*, 2012; El Khoury *et al.*, 2012). Recently, EFSA released a positive opinion on claims regarding effects of β -glucans in lowering post-prandial glucose and cholesterol levels (EFSA, 2011). Regarding effects on cholesterol levels, a minimum daily intake of 3 g of β -glucans from oats and/or barley is recommended to provide these health benefits, while regarding effects on post-prandial glucose, this claim can be used only for foods containing a minimum amount of 4 g of β -glucans from oats and/or barley with respect to 30 g of available carbohydrates in a meal (EFSA, 2011).

Non-dairy functional foods

Probiotics and prebiotics can be used as ingredients in the development of new functional products. From an historical perspective, dairy products represented the most widespread vehicle for the incorporation of probiotics in foods. In recent years, the food industry interest has expanded to non-dairy products as an alternative to traditional ones, such as foods derived from cereals (Gobbetti *et al.*, 2010). The incorporation of probiotic bacteria in cereal-based products represents a new challenge for the food industry. In fact, technological processes of food production should guarantee the stability and viability of added probiotics. Furthermore, the maintenance of the probiotic strain at high level during storage has to be assessed, together with the preservation of food sensory properties and nutritional values (Charalampopoulos *et al.*, 2002).

From a nutritional point of view, cereals represent appealing products for the development of functional foods since they are rich in components, i.e. fibre, proteins, minerals and vitamins, necessary for human health (Charalampopoulos *et al.*, 2002). Moreover, the non-digestible components of cereals can act as a prebiotic, constituting the substrate for the growth of microorganisms present in the colon. In particular, whole grain cereals can exert positive effects in protection against chronic human diseases such as cardiovascular disease, cancer, diabetes and obesity (Tuohy *et al.*, 2012). Fibre and polyphenols bound to the fibre constitute the two main classes of bioactive compounds of whole grain that can interact with the gut microbiota, with fibre representing the main energy source for colonic fermentation (Tuohy *et al.*, 2012), able to stimulate the growth of specific bacterial groups, e.g. *Bifidobacterium* spp. (Cooper *et al.*, 2015).

The gut microbiota: characteristics and functions

The human body harbours a complex and dynamic consortium of at least 100 trillion microorganisms (Jones *et al.*, 2014), considered in the past to exceed the number of human cells in proportion 10:1, and now, basing on recent estimation, evaluated to be in a ratio of about 1:1 (Sender *et al.*, 2016). Altogether the microorganisms residing inside the human body and on its surface constitute our microbiota, while the set of genes that they encode is defined microbiome and can participate in different functions that are essential for the host (Jones *et al.*, 2014).

The most broadly colonised organ is the gastrointestinal tract, in which the colon alone is estimated to account for over 70% of all the microbes in the human body (Sekirot *et al.*, 2010), with a concentration of about 10^{11} CFU/ml (Sender *et al.*, 2016). Hundreds to thousands species reside in the adult intestinal microbiota, mainly belonging to the Firmicutes and Bacteroidetes phyla (Human Microbiome Project Consortium, 2012). Firmicutes include genera *Clostridium*, *Enterococcus*, *Lactobacillus* e *Ruminococcus*, while genera *Bacteroides* and *Prevotella* belong to phylum Bacteroidetes. Other gut representative phyla are Actinobacteria, that includes genus *Bifidobacterium*), Proteobacteria and Verrucomicrobia (Sekirot *et al.*, 2010). A variety of distinct microbial habitats occur from the small intestine to the colon, due to chemical and nutrient gradients, as well as community differences are present over the cross-sectional axis of the gut (Donaldson *et al.*, 2016).

The adult gut microbiota is considered as relative stable, since a core of approximately 40 bacterial species, that represents about 75% of the gut microbiota in terms of abundance, persists for at least 1 year in individuals (Martínez *et al.*, 2013b), and during 5 years approximately 60% of microbiota composition in an individual is maintained (Faith *et al.*, 2013).

Considering numbers of total species found in gut microbiota, and average core species per individual, inter-individual variability in the composition of the microbiota can be particularly pronounced. In the perspective of highlighting a sort of phylogenetic core shared among individuals, a recent study has proposed a classification of the human gut microbiota in three different “enterotypes”, each of them characterised by the predominance of a certain bacterial genus, and in particular, *Bacteroides*, *Prevotella*, and *Ruminococcus* (Arumugam *et al.*, 2011). However, since it has been discussed how this classification could provide an oversimplified vision, a continuum or “gradient” of species has been subsequently proposed (Jeffery *et al.*, 2012). In addition, metagenomics studies suggested that, rather than a phylogenetic core, individuals share a functional core (Human Microbiome Project Consortium, 2012; Power *et al.*, 2014). In fact, a high degree of functional redundancy among resident microorganisms in the gastrointestinal tract has been observed (Louis *et al.*, 2010). As a results, most of the activities and effects of gut microbiota are not strictly dependent on the content in species, but derive from the presence of a diversified microbiota (Human Microbiome Project Consortium, 2012).

The gut microbiota exert a broad range of different functions that contribute to host physiology. One of the primary activities of resident microbiota consist in the fermentation of non-digestible carbohydrates, with parallel energy production (Flint *et al.*, 2012). Thanks to the metabolic activity of the microbial community, the human body is able to have access to the calories contained in the non-digestible polysaccharides of the diet; in fact, commensal bacteria can degrade these complex carbohydrate substrates in short-chain fatty acid (SCFA) and volatile substances (e.g. carbon dioxide, hydrogen) (Flint *et al.*, 2012). Acetate, propionate and butyrate represent important substrates for maintaining the integrity of the intestinal epithelium (Jones *et al.*, 2014). In particular, butyrate is the preferred energy source used by colonocytes, it is involved in the regulation of colonocyte differentiation, in the maintenance of mucosal integrity, and in the modulation of intestinal inflammation (Conlon and Bird, 2015).

Acetate and propionate enter into the systemic circulation and reach the liver and peripheral organs, where they are substrates for gluconeogenesis and lipogenesis (Tremaroli and Bäckhed, 2012). The energy obtained from the breakdown of carbohydrates by the intestinal microorganisms can become crucial in the case of metabolic syndrome including obesity; in this condition, an increase in SCFA production was reported (Teixeira *et al.*, 2012). The synthesis of short-chain fatty acids, defined saccharolytic fermentation, causes a lowering of the intestinal pH, thus representing an efficient defence system against pathogenic microorganisms (Flint *et al.*, 2012).

The intestinal bacterial community has also an important function in the development and regulation of the host immune system. In fact, bacterial colonisation is necessary for the structural and functional development of the immune system associated with the intestinal mucosa (gut-associated lymphoid tissue, GALT). Studies in germ-free animals showed an underdeveloped mucosal immune system, e.g. reduced gut secretory IgA and defects in gut-associated lymphoid tissue development (Jones *et al.*, 2014). In addition, commensal gut microbiota represent an important line of resistance against colonisation of exogenous microorganisms and also of pathogens, through competition for intestinal epithelial receptor sites and substrates, and production of antimicrobial substances (Sekirov *et al.*, 2010).

Finally, gut microbiota is involved in the production of vitamins, metabolism of steroids and cholesterol through the transformation of bile acids, metabolism of polyphenolic compounds and activation of bioactive compounds (Conlon and Bird, 2015; Sekirov *et al.*, 2010; Tremaroli and Bäckhed, 2012).

In most cases, studies about gut microbiota are performed on fecal samples, that require a easy, not invasive collection procedure, and represent an acceptable approximation of colonic microbiota, primarily because they are representative of the inter-individual variability (Brahe *et al.*, 2016). A broad range of techniques are available for exploring microbiota diversity, most of them relying in culture-independent approaches; this methods are less laborious and time-effective than conventional culture-dependent approaches, since nucleic acids are directly isolated from gut/fecal matrices, with no prior isolation (Fraher *et al.*, 2012). These techniques are based, in a large measure, on the analysis of the sequence of the 16S rRNA gene, and provide information about the microbial diversity of a sample, and/or the amount of specific bacterial groups. Among these, techniques aimed at revealing and quantifying specific target, defined *a priori*, are available, e.g. quantitative real-time PCR (qPCR). In principle, these kind of analysis can be carried out at all phylogenetic levels, according to the primer pair used. Complex communities can be investigated with fingerprint techniques, e.g. denaturing gradient gel electrophoresis (PCR-DGGE), or with high-throughput techniques, e.g. 16S rRNA gene profiling, that can provide a complete overview of the composition of studied populations, with no pre-defined target. Finally, the gold standard of culture-independent analysis is represented by the WMS (whole-metagenome sequencing), which allows to completely characterise samples with respect both to composition and function (Fraher *et al.*, 2012).

Influence of the diet on gut microbiota

Even considering the general stability of gut microbiota, transient environmental perturbations caused by dietary changes, antibiotic use, or diseases can result in significant variations in microbial populations of the gut ecosystem (Jones *et al.*, 2014).

In particular, diet represent one of the key factor in gut microbiota modulation. Numerous studies in recent years attempted to clarify the effects that diet can have on the intestinal communities, at different levels, from dietary patterns to consumption of specific foods or food components (Graf *et al.*, 2015). For example, regarding dietary pattern-associated microbiota, individuals with a diet characterised by a high protein intake were associated with a *Bacteroides*-type community, while individuals with a plant-based diet, i.e. a higher fibre intake, showed a *Prevotella*-type community (Wu *et al.*, 2011). In another study, analyses of fecal microbiota from Italian and African children showed how composition differed relevantly in the two groups, e.g. *Prevotella* spp. were more abundant in the African children than in the Italian children, in association with a higher fibre intake (De Filippo *et al.*, 2010).

Among various dietary patterns, the Mediterranean diet (MD), recognised from UNESCO as an intangible cultural heritage, is considered as one of the healthiest diets, because associated with a lower risk of incidence of certain diseases such as cancer, cardiovascular diseases and metabolic diseases including diabetes and obesity (Del Chierico *et al.*, 2014). Studies on faecal samples from individuals following this diet showed a higher concentration of short-chain fatty acids, and increased levels of *Prevotella* (De Filippis *et al.*, 2015).

Beside long-term dietary patterns, modulation of gut microbiota following consumption of different foods (e.g. whole grain, fruits and nuts, vegetables and legumes) or specific food ingredients (e.g. fiber/carbohydrates, fat, protein) have been also carried out (Graf *et al.*, 2015; Portune *et al.*, 2017).

Microbiota can quickly and dramatically change in composition and gene expression following switch to a different dietary pattern, e.g. from a plant- to an animal-based diets (David *et al.*, 2014), or in response to a specific diet component, e.g. resistant starch or non-starch polysaccharides, within a couple of day (Walker *et al.*, 2011). However, dietary interventions probably have a greater impact on the functionality than on the composition of the microbiota *per se*. In fact, short-term dietary interventions do not profoundly change the microbiota composition, but the microbial gene expression and the functional profiles seem to rapidly adapt to changes in diet (Graf *et al.*, 2015).

Dysbiosis condition and pathologies

In a healthy status, the human host lives in a state of natural balance with the inhabitants of his gastrointestinal tract. This homeostasis is maintained despite inter-individual variations in microbial composition, as well as short-term intra-individual fluctuations in response to environmental factors. This balance can be disrupted, and quantitative and qualitative changes at the level of microbial communities can affect their metabolic capacity leading to a condition of dysbiosis. Dysbiosis can derive from antibiotic use, diet, or can be related to different diseases, including autoimmune disease, inflammatory bowel diseases (IBD), obesity, diabetes (Jones *et al.*, 2014).

One of the common trends observed in dysbiosis is the reduction in microbial diversity, that has been reported in the context of metabolic syndrome (Le Chatelier *et al.*, 2013), IBD, elderly patients with inflammation, individuals after oral antibiotic treatment, type 1 diabetes (Jones *et al.*, 2014; Sanz *et al.*, 2015). If the dysbiosis of gut microbiota represent the cause or the consequence of a certain pathological condition has not been definitively clarified.

Dysbiosis conditions have been observed at different taxonomic levels, from phyla to species, e.g. from a low Bacteroidetes and Firmicutes ratio, to a low *F. prausnitzii* level in obesity (Turnbaugh *et al.*, 2009; Walters *et al.*, 2014). However, in a broad taxonomic group, for example, a genus, different species can be associated to a healthy or a pathological state (Arbolea *et al.*, 2016). In general, it has been proposed that complex phenotypes are associated with a dysbiotic microbiota, rather than a single disease-causing microbe (Jones *et al.*, 2014).

Another common trait in metabolic disorders/pathologies connected to a dysbiosis condition is represented by the modulation of intestinal permeability, that has been reported in several studies. For example, increased intestinal permeability has been observed in subjects affected by Type 1 diabetes (Vaarala *et al.*, 2008) and obesity (Teixeira *et al.*, 2012), in association with gut microbiota aberrations and to an inflammatory status.

Perspectives in restoring a healthy condition through the diet

Knowledge of the role that the gut microbiota plays in diseases and pathological condition is necessary, and it could be used to develop intervention strategies to prevent and/or treat imbalances that contribute to autoimmune and metabolic disorders (Sanz *et al.*, 2015; Brahe *et al.*, 2016). In fact, given the impact of diet on the composition and activity of gut microbiota, a specific dietary intervention could be useful in the modulation of a “dysbiotic” microbiota towards a “healthier” state.

With regard to the intestinal compartment, positive effects of a probiotic/prebiotic, or, in general, of an intervention could rely on the decrease of pathogen or pathobiont microorganisms (Sgouras *et al.*, 2004; Honda *et al.*, 2011), increase in bacterial diversity (Karlsson *et al.*, 2010), increase of beneficial resident bacterial groups, for example *Bifidobacterium* species (Bosscher *et al.*, 2009), or butyrate producer species, e.g. *Faecalibacterium prausnitzii* (Miquel *et al.*, 2014), and on reduction of gut permeability thanks to increased butyrate production (Brown *et al.*, 2011) or other mechanisms, e.g. influence on the tight junctions (Ahrné and Hagslätt). The strategy of improving health status through the use of probiotics, prebiotics, or functional foods have been explored in an increasing number of clinical trial dealing with metabolic syndrome or pathological conditions, with some positive microbiological and clinical outcomes (Iannitti and Palmieri, 2010; McFarland, 2014; Delzenne *et al.*, 2013, Jones *et al.*, 2014). For example, in the contest of obesity, treatment with different types of dietary fibres including prebiotics have been shown to modulate gut composition and improve insulin sensitivity, low-grade chronic inflammation, and lipid metabolism (Brahe *et al.*, 2016).

For investigating the impact of a dietary intervention on the gut microbiota, human trials represent the gold standard. However, clinical trials are expensive and difficult to control, mainly because of the fact that, often, participants differ markedly in their dietary behaviour and lifestyle (Graf *et al.*, 2015). Thus, an initial assessment performed through the utilisation of *in vitro* and animal models can provide preliminary useful information (Williams *et al.*, 2015). *In vitro* gut models represent a quick, easy and cost-effective tool of studying gut microbiota. Different categories of systems are available, and in general they also present some limitations, mainly related to the fact that *in vitro* models lack epithelial and immune cells, and feedback mechanisms by the host (Venema and Van den Abbeele, 2013). However, the use of such systems can allow to obtain mechanistic insight in understanding human colon microbiota, in terms of composition and metabolism in response to tested substrates, e.g. probiotics, prebiotics (Venema and Van den Abbeele, 2013; Williams *et al.*, 2015). Obtained knowledge can be then used for the planning of a clinical trial, that will finally assess the impact of the nutritional treatment at the systemic level.

2. Thesis outline and objectives

This thesis is focused on the development of a novel whole grain functional pasta enriched with a spore-forming probiotic strain, *Bacillus coagulans* GBI-30, 6086 (BC³⁰), and β -glucans from barley, from the first steps of the design and production, till the evaluation of its properties and potential effects on gut microbiota, and in general, on the consumer. The thesis is structured in three sections, the first one dealing with the development and characterisation of the functional pasta product, with specific reference to microbiological aspects related to the probiotic strain, while the second and the third part regard the investigation of the impact of the functional pasta on gut microbiota in two case contest, pre-diabetes/diabetes and overweight/obesity, respectively. Finally, overall concluding remarks are presented.

In **Chapter 1**, beside providing synthetic information on the production and characterisation of the overall pasta product, a specific relevance is given to the assessment of the stability of the probiotic strain during the pasta manufacturing and cooking processes; in fact, a probiotic bacterium has to be present at sufficiently high amounts in a dietary supplement or food, in order to guarantee certain doses during ingestion. Moreover, the stability during the transit trough the gastrointestinal tract (GI) represents another crucial point, since only if the probiotic strain is able to reach the colon alive it could exert its properties on the host. Thus, the survival of *B. coagulans* BC³⁰ while passing through the GI tract is investigated in **Chapter 2**.

Once that a new product has been developed, the most interesting question raises about which are the effects that it can induce in the host. Type 1 diabetes and obesity are two conditions in which alterations in gut microbiota have been reported. Since diet represents one of the most important factors involved in the modulation of gut microbiota, the restoration of a balanced microbiota condition through the use of specific dietary products is an interesting, not invasive strategy that is receiving much attention and appears as promising. In the contest of diabetes, a case-control study involving children at risk for type 1 diabetes (T1D) and healthy children has been firstly performed for investigating possible differences in microbiota composition, as described in **Chapter 3**. From these children, a small group has been individuated for a second study, reported in **Chapter 4**, that is specifically aimed at revealing the effects of the functional pasta on gut microbiota. This research is conducted using an *in vitro* model of the proximal colon, TIM-2, inoculated with fecal microbiota from the former children. In this study, not only the functional pasta is investigated, but also the functional ingredients *B. coagulans* BC³⁰ and β -

glucans are objects of study, in order to unravel their contribution in the pasta-related effects, and also expanding available knowledge about these specific products. Finally, following the preliminary *in vitro* step, the effects of the ingestion of the functional pasta on resident gut microbiota are assessed *in humana*, in a clinical trial involving overweight/obese adults, illustrated in **Chapter 5**. Moreover, in the perspective of disclosing the impact of the new functional product on the host, microbiological data are correlated with clinical results, in an integrated view.

First part

Chapter I

Development of a novel functional pasta containing β -glucans and a stable probiotic strain, *Bacillus coagulans* GBI-30, 6086

1.1. Introduction

During the last decade, consumer requirements in the field of food production have changed considerably. Thus, foods are no more intended to only satisfy hunger and to provide the necessary nutrients, but also to prevent nutrition-related diseases and to improve physical and mental well-being. In this frame, the functional foods represent one of the most interesting areas of research and innovation in the food industry.

Cereals can be considered important sources of functional ingredients as they contain, especially in the outer layers of kernels, substances (i.e. polyphenols) with protective activities related to the reduction in the incidence of coronary heart disease, diabetes and cancer (Hemery *et al.*, 2007). Most of the bioactive compounds are linked to fibre and are present in bran, while most of vitamins E and B, minerals and sterols are present in the germ. However, the production of foodstuff from whole grain must guarantee to the consumer the absence of mycotoxins and pesticide residues. A good compromise between food security and preservation of the main health compounds of wheat is represented by the application of the debranning process prior to milling, for which new processes have been developed (Hemery *et al.*, 2007; Fares *et al.*, 2010).

To enhance the healthy properties of semolina, barley flour is often added to increase the soluble fibre, mainly the β -glucans content. Due to their chemical and physical properties, β -glucans, which can form high-viscosity solutions, could aid in reduction of blood cholesterol levels and lower glycemic response (Tosh, 2013), as also recently recognized by EFSA (EFSA, 2011).

Probiotics are “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” (FAO/WHO, 2002; Hill *et al.*, 2014). Many different microorganisms have been used as probiotics, mostly strains of the bacterial genera *Lactobacillus* and *Bifidobacterium*, but also strains of *Escherichia coli*, *Saccharomyces boulardii* or *Bacillus coagulans*, together with other spore-forming bacilli (Cutting, 2011). For their spore-forming nature, these latter organisms possess improved viability and stability compared to other

probiotic bacterial strains, e.g. the ability to withstand high temperature processes, such as baking and boiling, thus representing an ideal choice for the development of functional cereal-based products. Indeed, the endospores of the genus *Bacillus* are a very stable duration form and show high stability during the processing and storage of the food product (Bader *et al.*, 2012). Further, in many food products, germination of the spores does not occur and thus the quality of the product is not affected because of their inactive metabolism.

Some commercial probiotic formulations containing *B. coagulans* are actually available, including *B. coagulans* GBI-30, 6086 (marketed as GanedenBC³⁰, thereafter referred as BC³⁰), that has been also incorporated into foods, thanks to the fact that spores can survive manufacturing processes, including mild heat-treatments used to sterilise food (Cutting, 2011). This spore-forming strain has obtained the Generally Recognized As Safe (GRAS) status in 2012 from the U.S. Food and Drug Administration (FDA), following assessment of toxicological studies (Endres *et al.*, 2011). Moreover, the species *B. coagulans* is included in the list of Qualified Presumption of Safety (QPS) biological agents that can be added to food (EFSA, 2007). *Bacillus coagulans* BC³⁰ has been reported to improve the symptoms related to gastrointestinal disorders, such as irritable bowel syndrome and intestinal gas, and rheumatoid arthritis (Jurenka, 2012), and it has been shown to enhance the immunological response in common viral infections of the respiratory tract (Kimmel *et al.*, 2010). Furthermore, evidences from animal models show that *B. coagulans* BC³⁰ acts as a coadjuvant in the response towards pathogens in colitis (Fitzpatrick *et al.*, 2012).

The present study is aimed at the design and development of a novel functional pasta product, not yet present on the global market, formulated with durum wheat flour naturally enriched in phenolic acids, and added with *B. coagulans* BC³⁰ and barley β -glucans. A second pasta obtained from the same flour, but without addition of the two functional ingredients represents the respective control. In particular, the microbiological focus of the present study consists in the evaluation of the fate of the probiotic strain during pasta-making and cooking processes, using culture-dependent and culture-independent approaches. Therefore, information about the preparation and characterisation of the newly developed products will be briefly provided, in order to have a more comprehensive overview.

1.2. Material and methods

Preparation of durum wheat flours and pasta manufacturing

An Italian durum wheat (*Triticum durum* L.) cultivar (Vendetta) was grown in Foggia during 2010-2011 and used to evaluate on a small-scale the process for obtaining a flour enriched in phenolic compounds and fibre. The following year (2011/2012), the cultivation of Vendetta was carried out to start the industrial process (25 q). The durum wheat was milled by Antico Molino Rosso SRL (Buttapietra, VR, Italy), conducted in a first step by a debrannig process and subsequently by stone milling. The milled grain was divided into two batches. The first batch (12.5 q), consisting of durum wheat Vendetta hulled up to 4.5% and stone milled (control flour); the second batch (12.5 q), consisting of the durum wheat flour described above (78.5%) mixed and homogenized with 18% of enriched barley flour (11% β -glucans) (Agro Alimentare Sud, Melfi, Potenza, Italy), and 3.5% of vital gluten powder (Sedamyl S.p.A., Saluzzo, Cuneo, Italy). A small quantity of the second batch (500 kg) was added with 1% of a freeze-dried preparation of the probiotic culture described below (functional flour).

Two different shapes of pasta were produced on a small scale for preliminary analyses, and in particular: *spaghetti* with BC³⁰ added, obtained at different drying temperatures (50°C and 90°C), manufactured at the Agricultural Research Council (CRA) (Rome, Italy), and *fusilli* with BC³⁰ added, obtained at a low drying temperature (50°C), manufactured at the industrial plant Rustichella d'Abruzzo S.p.A.

Two novel pasta, one derived from the control flour (control pasta), and one derived from the functional flour (functional pasta) were then produced at the plant Rustichella d'Abruzzo S.p.A. The durum wheat flours were mixed with water to obtain a total dough water content of 41-42%, and, after mixing, the product was extruded into the shape of *penna rigata* pasta of 1.12 mm of thickness, 38-42 mm of length and 9 mm of diameter. Pasta products were dried at about 50°C.

Characterisation of pasta samples

Determination of total dietary fiber and β -glucans content, protein content, yellow pigment content, phenolic acids, and antioxidant activity were performed on the durum wheat flours and pasta samples (uncooked and cooked). Available carbohydrate, fat and ash contents were analysed with respect to uncooked and cooked pasta products. Cooking characteristics of pasta samples were determined, i.e. cooking time, pasta firmness, and texture parameters. The glycemic index of the pasta products was also evaluated. All detailed information about these analyses are available in the study of Fares and colleagues (2015).

Microbiological analyses

The strain *B. coagulans* BC³⁰, a patented probiotic bacterium widely marketed as a dietary supplement or incorporated in functional foods was selected to be used as the probiotic ingredient in the pasta-making process. The freeze-dried probiotic preparation was obtained from Sochim International S.p.A. (Cornaredo, Milan, Italy) and contained a declared concentration of 10.3 log CFU/g.

Preliminary analyses on different shapes of pasta, i.e. *spaghetti* with BC³⁰ added, obtained at drying temperatures of 50°C and 90°C, and *fusilli* with BC³⁰ added, obtained at a drying temperature of 50°C, were performed to assess the viability of the probiotic strain.

To evaluate the survival of the probiotic strain during the cooking process, pasta (*fusilli*) (10 g) was cooked in 100 mL of boiling tap water, for different cooking times (5, 7, 9 and 11 min). The effect of the addition of NaCl to the cooking water was also evaluated, as well as the dispersion of *B. coagulans* cells in the cooking water.

Regarding samples from the production chain of the novel functional pasta, the freeze-dried BC³⁰ preparation, the durum wheat flour added with barley flour (semolina), semolina with 1% of BC³⁰ added, the uncooked and cooked functional pasta, and the cooking water were analysed. The control pasta was analysed, too.

Enumeration of *B. coagulans* BC³⁰ was carried out by conventional plating following the recommendations of USP's Food Chemicals Codex (FCC) (2014). Regarding pasta sample preparation, dried samples were ground to homogeneity using a household blender, while cooked samples were homogenized with a Ultra-Turrax T25 (Janke and Kunkel, IKA-Labortechnik, Staufen, Germany); then, 1 g of sample was weighed out and suspended in 199 mL of sterile peptone (0.1%) water. Appropriate decimal dilutions were pour plated in duplicate in Glucose Yeast Extract (GYE) agar, before and after a heat-treatment (75°C for 30 min) step, and incubated aerobically at 37°C for 48 h; this last part of the procedure was followed for each sample analysed. The experiments were performed as biological replicates.

Furthermore, enumeration of the probiotic strain in the functional and control pasta was conducted by a qPCR assay. Initially, serial dilutions of the freeze-dried *B. coagulans* BC³⁰ dissolved in sterile peptone water were used to generate a standard curve. The cells were counted by plating and subjected to DNA extraction and qPCR analysis as described below.

DNA was extracted using the protocol of Marmur (1961) with some modifications. In particular, cells were harvested from 2 mL of the serial dilutions and lysed with 10 mg/mL lysozyme in Tris-EDTA pH 8 for 60 min at 37°C; moreover the DNA treatment with 20 µg/mL proteinase K

was performed for 30 min at 37°C. DNA concentration was determined using a NanoDropLite spectrophotometer (Thermo Scientific, DE, USA).

Quantitative PCR was performed in the Light Cycler Nano System (Roche, Mannheim, Germany). The primers B^G-For and B^G-Rev were designed on the 16S rDNA gene and their specificity for *B. coagulans* was assessed *in silico* and on a collection of *Bacillus* spp. strains.

The qPCR reactions were performed in a total volume of 20 µL containing Fast Start Essential DNA Green Master and PCR grade water (Roche, Mannheim, Germany), 0.5 µM of forward and reverse primers, and 5 µL of genomic DNA. The amplification program consisted of 1 cycle of 95°C for 10 min, followed by 45 cycles of 95°C for 15 sec, 66°C for 15 sec, and 72°C for 16 sec. The qPCR experiments were performed as biological replicates and technical triplicates.

The efficiency (E) of the qPCR reactions was calculated using LinRegPCR version 2012.3 according to Ruijter *et al.* (2009).

Isolation of DNA from pasta samples was performed starting from 2 mL of a suspension of homogenised pasta in sterile peptone water, and following the protocol of Cocolin *et al.* (2000) using 425-600 µm-diameter glass beads (Sigma, Milan, Italy). After cell disruption, an additional step with CTAB 2X (1% PVP, 2% cetyltrimethylammonium bromide, 100 mM Tris-HCl pH 8.0, 20 mM EDTA pH 8.0, 1.4 M NaCl) was performed at 65°C for 5 min and followed by a purification step with chloroform/isoamyl alcohol.

Quantification of the probiotic strain in the pasta samples was calculated based upon comparison of the corresponding threshold cycle (Ct) values with the standard curve.

Results are presented as means ± standard deviation (SD). The significance of the *B. coagulans* BC³⁰ count results was evaluated using Student's t-test. Differences were considered significant at $p < 0.05$.

1.3. Results and discussion

Preliminary analyses for assessing the viability of the probiotic strain during pasta-making and cooking processes

The viability of the probiotic strain *B. coagulans* BC³⁰ has been firstly assessed in a series of preliminary analyses, performed with different experimental pasta. These analyses were performed with plate counts.

First, analyses of the viability of the probiotic strain were conducted with reference to two different drying processes (Figure 1.1). In particular, a process at a low drying temperature, i.e. 50°C for 18 hours, and a process at a high drying temperature, i.e. 90°C for 4 and a half hours,

were tested. In this case a “long” pasta format, *spaghetti*, produced on a small scale, was analysed, dried and cooked. Cooking times of 7 and 10 minutes were tested for the analyses.

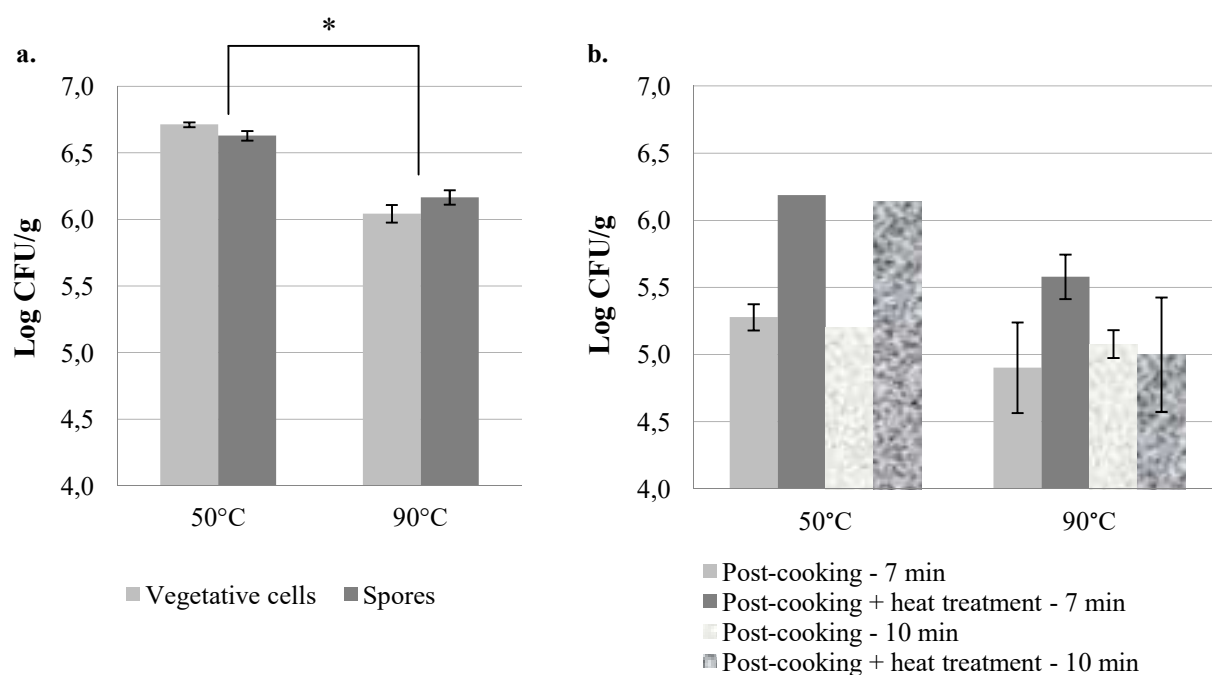


Figure 1.1. Quantification of the probiotic strain *B. coagulans* BC³⁰, in the uncooked (a) and cooked (b) experimental *spaghetti* pasta, following a low temperature (50°C) or a high temperature (90°C) drying processes. Regarding cooked pasta, analyses with two different cooking times (7 or 10 minutes), and performed by plating immediately after cooking, or after a subsequent heat treatment are presented.

*: $p < 0.05$.

Plate count analyses were performed on the uncooked pasta after grinding, and after the heat treatment. Results obtained before the thermal treatment provided information about amounts of vegetative cells and spores, while from the results obtained following the heat treatment the amount of spores were retrieved, in according to previous works (Maathuis *et al.*, 2010; Ghelardi *et al.*, 2015). In the uncooked product, dried with both processes, vegetative cells and spores were observed to be approximately in a 1:1 proportion (Figure 1.1a). However, a significant reduction of the microbial loads in the pasta dried at the higher temperature was observed. As a consequence, also the amount of probiotic cells that survived the cooking process was considerably lower in the pasta produced with the high temperature process (Figure 1.1b). Also for the analyses of cooked pasta, samples were plated immediately or after a following thermal treatment. In this case, considering that vegetative cells are expected to die during the cooking processes, results obtained immediately after cooking suggested that a quote of spore germinated proximately after the cooking process. Pasta dried at the low temperature exhibited the same amounts of spores (before or after the thermal treatment), independently from the cooking time.

Pasta dried at the high temperature exhibited a decrease in the amounts of spores, that appeared more marked in the case of 10 minutes cooking, as expectable. Moreover, in this case, average amount of spores counted after cooking corresponded to the amount of spores counted after the subsequent heat treatment, indicating that, probably, 10 minutes represented a sufficiently long period to induce germination of all present spores (Figure 1.1b).

Further analyses were then conducted to specifically evaluate the impact of different cooking times on the viability of the probiotic strain. In this case, analyses were performed on the experimental *fusilli*, a “short” pasta format, dried at 50°C, and that was cooked for 5, 7, 9 or 11 minutes. These analyses indicated that the cooking process caused a reduction of the bacterial counts that depended on the cooking time of the functional pasta. Indeed, similar cell numbers were observed applying cooking time of 5 min (6.9 ± 0.1 log CFU/g) and 7 min (6.8 ± 0.3 log CFU/g), while 9 and 11 min led to a significant ($p < 0.05$) decrease in the counts (6.4 ± 0.3 log CFU/g and 6.3 ± 0.1 log CFU/g, respectively). The addition of 1% NaCl to the cooking water did not have a negative effect on the viability of the probiotic strain (data not shown). A certain quote of probiotic cells were released from the pasta matrix in the water during cooking; however this quote did not represent a relevant amount (2-3 log CFU/g of pasta).

Results of these preliminary analyses showed that the probiotic strain withstood the pasta-manufacturing and cooking processes. In particular, the drying process at low temperature could guarantee a high viability of the probiotic strain, while the higher drying temperature had a detrimental effect. Moreover, a short cooking time (5 or 7 minutes) guaranteed a higher survival of the bacterial strain, suggesting that a pasta with an appropriate thickness, that can be cooked in few minutes, could represent an optimal solution to deliver the spore-forming probiotic strain. Considering these findings, the functional experimental pasta was produced on a large scale, in the format of *penne rigate*, which have a cooking time of approximately 6-7 minutes. Therefore, subsequent analyses were performed using cooking time of 5 and 7 min, and by adding NaCl to the water in order to better simulate the domestic cooking process.

Analyses of the novel functional and control pasta

Regarding the functional pasta produced at the industrial level, samples derived from the different steps of the pasta-making and cooking processes were analysed, i.e. the freeze-dried BC³⁰ preparation, the durum wheat flour added with the barley flour (semolina), the semolina with 1% of BC³⁰ added, the uncooked and cooked pasta, and the cooking water. Plate count analyses were performed on each sample without and with the thermal treatment, as reported in

Materials and Methods section. Quantification of the freeze-dried *B. coagulans* BC³⁰ preparation showed that before and after the thermal treatment the same number of cells were counted (Figure 1.2a); thus, the found BC³⁰ amount was considered to be 100% of spores, and it corresponded to the expected cell concentrations, i.e. approximately 10.2 log CFU/g (Figure 1.2b).

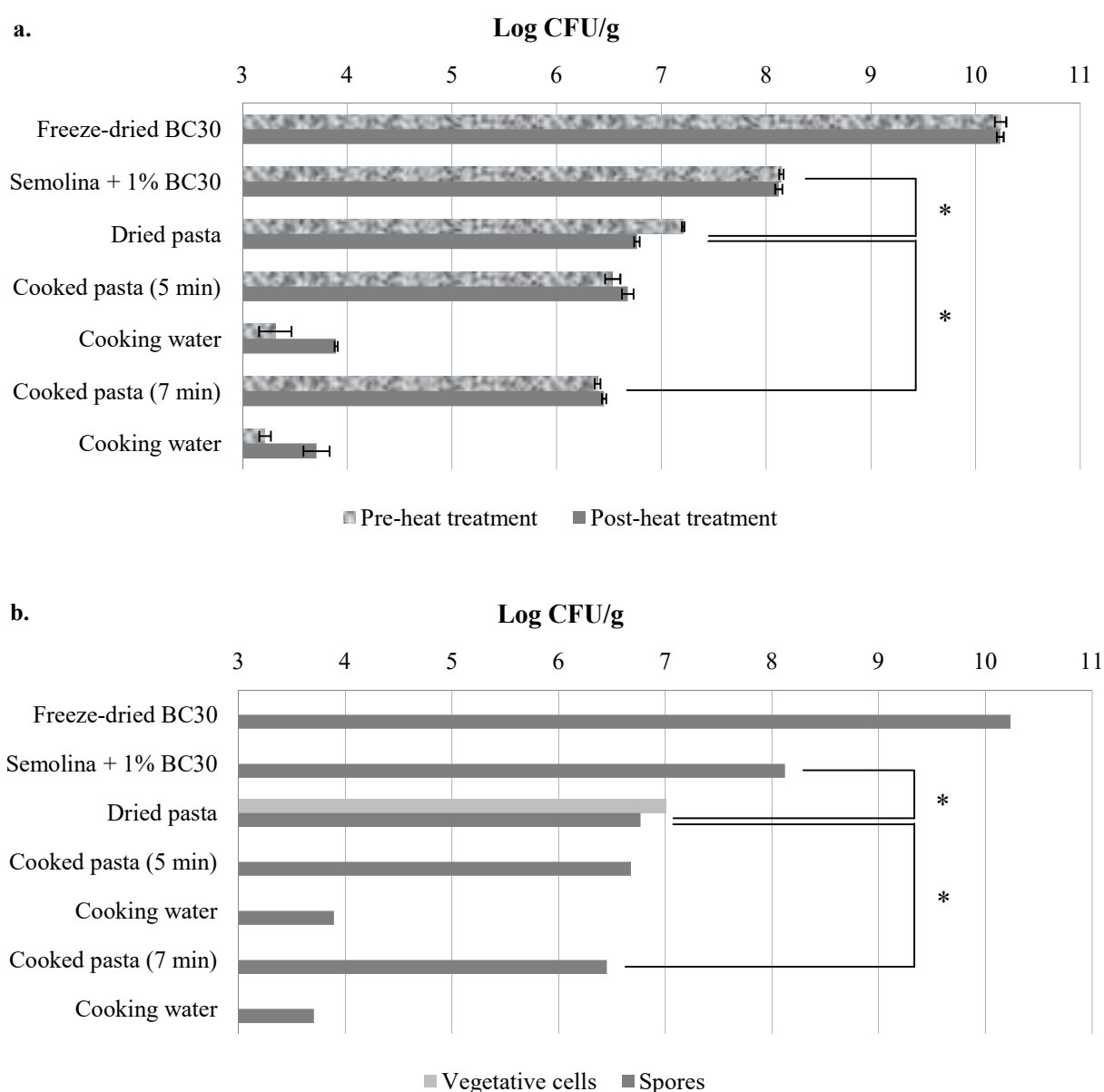


Figure 1.2. Quantification of the probiotic strain *B. coagulans* BC³⁰, in the freeze-dried BC³⁰ preparation, in the semolina with 1% of BC³⁰, in the dried and cooked *penne rigate* pasta, after 5 or 7 minutes cooking, and in the cooking water (BC³⁰ amounts in the cooking water are expressed in CFU/ g of pasta). Results are expressed as a) values obtained prior or after the heat treatment and as b) estimations of vegetative cells and spores amounts. *: $p < 0.05$. Statistically significant differences were indicated for comparisons among semolina with 1% BC³⁰ added and dried and cooked pasta samples.

Observed colonies were small (1-5 mm in diameter for surface colonies, 0.5-1 mm for colonies inside the agar), white to cream coloured; surface colonies were round and convex with smooth edges, while colonies inside the medium appeared as small points (Figure 1.3), as reported in USP, 2014.

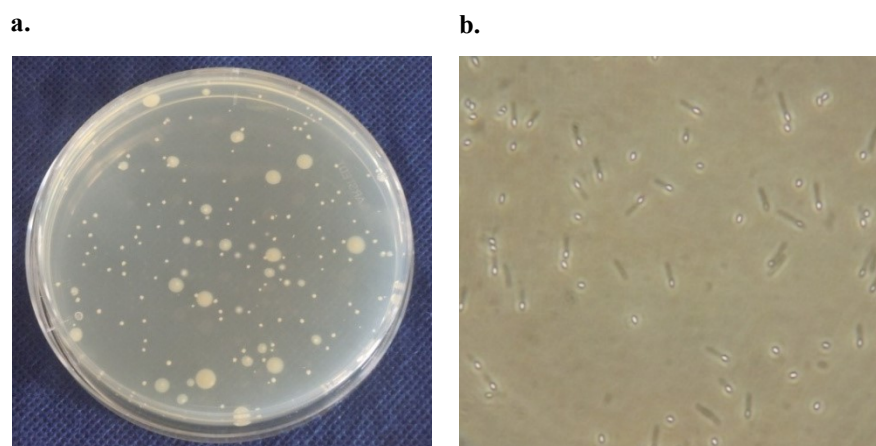


Figure 1.3. *B. coagulans* BC³⁰, as observed from plating analyses (a) and at the optical microscope (b).

Parallel to the quantification of the probiotic product, also the durum wheat flour added with barley flour (semolina) used for the production of the functional pasta was analysed, in order to evaluate the presence of environmental contaminants belonging to the genus *Bacillus*. Plate count analyses showed a spore load of approximately 4 log CFU/g, indicating that the flour was contaminated with environmental spore-forming bacilli, in agreement with previous evidences (Fangio *et al.*, 2010; Sorokulova, 2013).

B. coagulans BC³⁰ was then quantified in the semolina added with 1% of the probiotic strain. Also in this case, the same count values were obtained before and after the thermal treatment (Figure 1.2a), thus confirming the presence of 100% spores (Figure 1.2b). In particular 8.1 log CFU/g were found in the mix, in agreement with the amount added. In this contest, several samples deriving from different bags in which the product was collected were analysed, in order to evaluate the homogeneity of the dispersion of the probiotic strain in the flour. Results were highly reproducible, thus demonstrating the effectiveness of the mixing process.

Analyses of the uncooked functional pasta samples were carried out for monitoring the viability of the probiotic strain following the food production process, and indicated that *B. coagulans* BC³⁰ was present at a concentration above 7.0 log CFU/g (total of vegetative cells and spores) (Figure 1.2a). Thus, the general stability of the probiotic strain during the pasta-making process was confirmed, even if a significant reduction was observed with respect to the semolina with

BC³⁰ added. This was probably due to the difficulty in retrieving the bacterial cells from the hard, compact matrix of the dried pasta. Moreover, in the dried pasta, a high level of presumed vegetative cells was observed, that could be caused by a germination process occurred during the step of water addition to semolina. However, further studies would be necessary to clarify this aspect.

Regarding the analyses of the cooked functional pasta samples, results on the *penne rigate* confirmed previous preliminary data on the *fusilli*. The cooked pasta included more than 6.0 log CFU/g and no differences in BC³⁰ amounts between cooking for 5 or 7 minutes were found (Figure 1.2a and Figure 1.2b). However, a significant difference between the dried pasta and the pasta cooked for 7 minutes was found (Figure 1.2a and Figure 1.2b), indicating that a certain reduction in BC³⁰ amounts occurred during 7 minutes cooking. Finally, a release of about 4.0 log CFU/g of pasta was observed in the cooking water (Figure 1.2a and Figure 1.2b), corresponding to approximately 3.0 log CFU/ml of water. In the case of the cooked samples (pasta and water), results obtained from plating prior to the heat treatment could represent a quote of spores that germinated just after the cooking process, as previously observed for the *spaghetti*.

Also with regard to the control pasta, plate count analyses were performed to assess the presence of spore-forming bacteria. Results on dried pasta samples showed values of about 3.5 log CFU/g spores, confirming the presence of environmental contaminants belonging to the genus *Bacillus*. However, these bacteria were not revealed anymore in the cooked product, thus indicating that they did not survive the cooking process.

To fulfill the requirements of a robust quality management, a qPCR assay based on 16S rRNA gene for accurate identification and quantification of *B. coagulans* BC³⁰ was developed.

The newly designed primers B^G-For (5'-AAAAGCTTGCTTTTAAAGGTTAGCG-3') and B^G-Rev (5'-GTTCGAACGGCACTTGTCT-3') provided an amplicon of 400 bp that was demonstrated to be specific for this species. Indeed, using the BLAST algorithm, used primers were confirmed to target *B. coagulans* sequences. Thus, the selected 16S rRNA gene region appears to be a suitable target for the identification of the probiotic strain. Further, no amplification was observed when purified DNA from other spore-forming bacterial species was used, supporting the specificity of this novel PCR procedure.

To correlate amplification curves and C_T values with colony forming units and the actual number of cells present in one sample, a standard curve was produced from DNA extracted from 10-fold dilutions of the freeze-dried BC³⁰ product, as described in the Materials and Methods section. A linear response was observed over a range of five order of magnitude (R²= 0.990), and the limit

of detection was 3.0 log CFU per assays. The efficiency of the qPCR reaction was 1.88, according to Ruijter *et al.* (2009). From the standard curve, the level of *B. coagulans* was calculated using the equation $\log \text{CFU} = (\text{Ct} - 41.36) / -4.79$.

One of the key advantages of this assay is its rapidity, as it allows specific detection of *B. coagulans* within a day without any prior cultivation. In contrast, culture-dependent techniques, such as conventional colony PCR plus sequencing needs up to 96 hours to identify the isolated bacteria at the species level.

B. coagulans enumeration by qPCR was performed on dried pasta samples and on 5 minutes and 7 minutes cooked pasta samples, providing values of $7.5 \pm 0.5 \log \text{CFU/g}$, $7.2 \pm 0.2 \log \text{CFU/g}$, and $7.3 \pm 0.2 \log \text{CFU/g}$, respectively. The qPCR data were slightly higher compared to the results from plate counts probably due to the presence of dead cells or ungerminated spores, which contributed to the signal in the molecular analysis. However, *B. coagulans* BC³⁰ total levels resulted above 7.0 log CFU/g with both techniques, supporting the fact that the industrial pasta-making process had no strong detrimental effect on the viability of the probiotic bacteria. The cooking process caused a small reduction of *B. coagulans* BC³⁰ concentration, but no difference between samples cooked for 5 or 7 min was observed, consistently with plate analyses. Regarding the control pasta, contaminants observed through plate count analyses were not detected by the qPCR assay, indicating that they probably belonged to other *Bacillus* species, different than *B. coagulans*.

Another test was performed by cooking the pasta (for 7 minutes), storing it into the fridge overnight, and warming it up into the microwave oven the following day, in order to simulate what can be commonly done by a consumer, and to verify the viability of the probiotic strain in this condition. Results showed that this procedure did not affect the viability of the probiotic strain. In fact the same level of the probiotic strain, i.e. 6.2 log CFU/g of pasta, was found after plating the sample after the overnight conservation and the following warming up, or immediately after pasta cooking.

Stability of the probiotic strain during functional pasta storage

The stability of the probiotic strain during long-term storage of the functional pasta was evaluated for *fusilli* and *penne rigate* pasta samples. Analyses were performed on samples stored at room temperature (approximately 20°C-22°C), for several months. Quantifications were

performed on cooked pasta, since this represent the most interesting data defining the actual amount of bacteria that can be ingested.

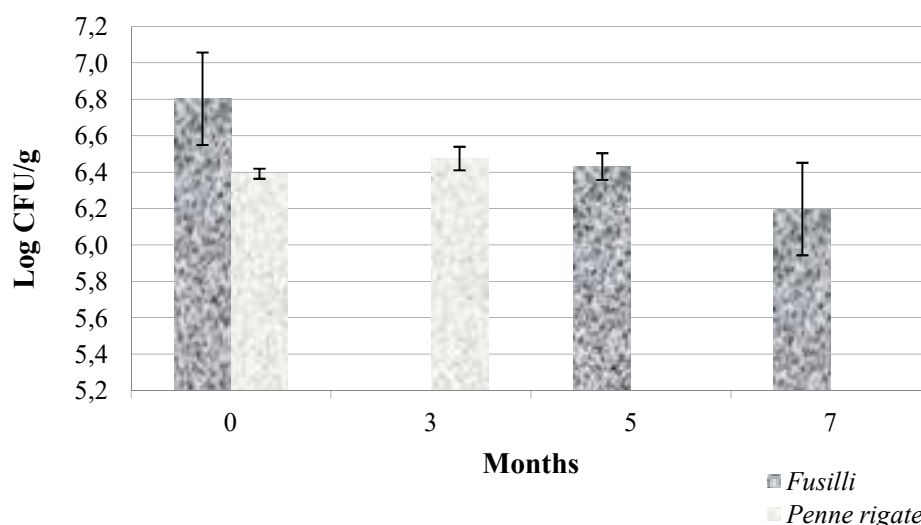


Figure 1.4. Quantification of the probiotic strain *B. coagulans* BC³⁰, in the *fusilli* and in the *penne rigate* cooked samples, after storage from several months. Cooking was performed for 7 minutes.

Results showed that for both pasta, the amount of the probiotic strain was kept at high levels, i.e. more than 6.0 log CFU/g of pasta, for several months, till to half a year (Figure 1.4).

Another study in literature assessed the viability of a *B. coagulans* strain added to different foods after storage for several months, i.e., 1, 2, 3, 6 and 12 months (Majeed *et al.*, 2016). Also in this work, the viability of the probiotic strain was shown to be extremely high, both with storage in frozen conditions (e.g. waffles samples) or at room temperature (e.g. chocolate fudge frosting).

General characterisation of durum wheat flour and pasta samples

The flour samples were characterised by a high protein content (PC) of approximately 18%. Since PC has a crucial role in determining the cooking performances of pasta products (Del Nobile *et al.*, 2005), a great relevance was assigned to this result, obtained through the debranning process. Moreover, as a result of the cooking process, in both control and functional *penne rigate* samples, PC increased. Furthermore, obtained wheat flours resulted enriched in phenolic acids, further demonstrating the effectiveness of the debranning process prior to milling (Fares *et al.*, 2010).

The addition of β -glucans from barley significantly increased the amount of total dietary fibre (TDF) in the functional flour, in agreement with the amount of β -glucans added. According with previous findings (Fares *et al.*, 2008; Fares *et al.*, 2010), fibre and β -glucans content was

positively affected by cooking, and in the two cooked product the amounts of TDF were comparable. The main event responsible for these observed increases of TDF is the gelatinisation and retro-degradation of the starch during cooking, which changes part of it into resistant starch and leads to an increase in the TDF (Vasanthan *et al.*, 2002). β -glucans were found at a final concentration of 2.3% in the dried functional pasta, and of 2.6% in the cooked one.

Regarding the glycemic index (GI) evaluation, all GI values resulted medium, with no statistically significant differences between analysed pasta, indicating that the addition of the functional ingredients did not affect the glycemic response.

Detailed information on all characterisation performed on flours and uncooked and cooked pasta are reported in Table S1.1, S1.2, and S1.3.

2.4. Concluding remarks

A new functional pasta obtained from a durum wheat flour rich in phenolic acids, and with added *B. coagulans* BC³⁰ and β -glucans from barley was developed. This new pasta product combined the health potential of cereals that are further strengthened by the presence of a probiotic strain, in a stable, storable product, that could represent a high-quality food for its nutritional appeal as well as the potential beneficial properties.

Regarding the probiotic strain *B. coagulans* BC³⁰, preliminary analyses provided indications about resistance of the strain at two different pasta drying processes, and led to define the optimal pasta cooking time in order to maintain a high viability. Then, the functional pasta produced on the industrial scale was analysed, and the viability of the probiotic strain during the manufacturing process and in the subsequent cooking step was demonstrated using both culture-dependent and culture-independent methods. Thus, considering that the cooked functional pasta included more than 10^6 CFU of the probiotic strain per gram, for an average portion of 70-100 g of pasta consumed during a meal, a minimum dose of approximately 10^8 CFU of probiotic bacterium *B. coagulans* BC³⁰ would be introduced with the diet. Given the high stability of the spore-forming bacterium, it is supposed that the probiotic strain could also be able to withstand the transit through the stomach to the intestine, as previously demonstrated in an *in vitro* study (Maathuis *et al.*, 2010), thus being potentially able to exert its positive properties.

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Supplementary material

Table S1.1. Functional properties of the control and functional durum wheat flours, obtained by Vendetta variety.

Durum wheat flour samples	PC	YPC	TDF	β-glucans	Phenolic acids		Antioxidant activity	
	(%)	(%)	(%)	(%)	(μg/g)		(μmoli trolox /g)	
					Free	Bound	Free	Bound
Control flour	18.18±0.19 a	3.77±0.01 a	12.63±0.07 b	0.36±0.01 b	2.73±0.38 a	389.4±14.71 a	1.36±0.21 a	12.94±0.27 a
Functional flour	17.72±0.03 a	3.78±0.03 a	13.61±0.13 a	2.64±0.03 a	2.04±0.07 b	364.8 ±5.66 a	1.41±0.13 a	13.46±0.94 a

Data are means of three determinations ± standard deviation. Different letters in the same row indicate statistical differences by LSD test ($p < 0.05$).

Abbreviations: PC = protein content; YPC = yellow pigment content; TDF = total dietary fibre.

Table S1.2. Functional properties of the uncooked and cooked control and functional pasta samples.

Pasta samples	PC	YPC	TDF	β-glucans	Phenolic acids		Antioxidant activity	
	(%)	(%)	(%)	(%)	(μg/g)		(μmoli trolox /g)	
					Free	Bound	Free	Bound
Uncooked								
Control	19.22±0.01 b	3.63±0.03 a	12.52±0.03 d	0.37±0.01 e	2.62±0.40 b	499.97±23.58 a	0.79±0.01 b	12.70±0.09 ab
Functional	16.84±0.04 f	3.26±0.03 c	13.63±0.33 c	2.30±0.01 d	2.46±0.13 b	421.14±8.38 bc	1.19±0.02 a	11.44±0.15 c
Cooked								
Control	19.81±0.16 a	2.74±0.03 d	13.57±0.15 c	0.42±0.00 e	2.32±0.04 b	504.99±22.64 a	0.77±0.06 b	13.44±0.21 a
Functional	17.29±0.00 e	2.37±0.01 f	13.80±0.05 c	2.61±0.04 a	2.08±0.07 b	401.74±4.63 c	0.72±0.22 b	12.90±0.49 ab

Data are means of three determinations ± standard deviation. Different letters in the same row indicate statistical differences by LSD test ($p < 0.05$).

Abbreviations: PC = protein content; YPC = yellow pigment content; TDF = total dietary fibre.

Table S1.3. Proximate composition of uncooked pasta samples, cooking behaviour and glycemic index of cooked pasta samples.

Pasta samples	ACC(%)	Fat (%)	Ash (%)	CT (min)	WCP (%)	Firmness	GI
Control pasta	63.2 ± 1.8 a	2.67 ± 0.07 a	1.43 ± 0.01 b	7	100%	770 ± 29.0 a	67.5 ± 5.3 a
Functional pasta	61.5 ± 1.2 a	2.37 ± 0.05 a	1.51 ± 0.01 a	7	100%	782 ± 30.2 a	59.7 ± 6.0 a

Data are means of three determinations ± standard deviation.

Different letters in the same row indicate statistical differences by LSD test ($p < 0.05$).

Abbreviations: ACC = available carbohydrate content; CT = cooking time; WCP = weight cooked pasta; GI = glycemic index.

Chapter II

Survival of the probiotic strain *Bacillus coagulans* GBI-30, 6086 to the transit into the gastrointestinal tract

2.1. Introduction

Probiotics are frequently administered to improve gut functionality or restore a gut microbiota balance (Iannitti and Palmieri, 2010). In this perspective, one of the main requirements for a probiotic strain is to survive during the transit through the different compartment of the gastrointestinal (GI) tract (Papadimitriou *et al.*, 2015).

After ingestion, probiotic bacteria have to face a hostile environment; in particular, they have to survive the low pH of the stomach, below 3, and high levels of pepsin, which could lead to cell inactivation and death. Moving forward through the GI tract, pH rise to values above 6, but the presence of bile, pancreatin and lipase constitute another critical point. If survived to these conditions, once reached the colon, ingested bacteria are capable to transiently integrate the gut microbiota (Derrien and van Hylckama Vlieg, 2015).

In order to assess the survival of probiotics to the transit through the GI tract, quantification of the ingested strain in the fecal samples is usually performed, both with the use of culture-dependent or molecular approaches. Analyses of the fecal samples allow to define the so called “persistence”, that results from the balance among the dose of the ingested strain, the extent of cell death and replication of surviving cells (Derrien and van Hylckama Vlieg, 2015).

Spore-forming probiotic bacteria present improved stability and resistance than conventional probiotics. Because of these properties, *Bacillus* species have received an increasing attention as probiotics, and they are being extensively used (Cutting, 2011).

Regarding the species *B. coagulans*, few information about the resistance to low pH values and to the other adverse conditions present in the intestinal environment are available, and they indicate that these properties are strain dependent (De Vecchi and Drago, 2006). The survival of the probiotic strain *Bacillus coagulans* GBI-30, 6086 (thereafter BC³⁰) was investigated in an *in vitro* study (Maathuis *et al.*, 2010), and then confirmed in an *in humana* trial (Nyangale *et al.*, 2015). In the first study, survival and activity of the strain BC³⁰ in the first portion of the GI tract were assessed through the use of an *in vitro* model, representative of the stomach and small

intestine. Results showed that the 70% of spores survived, thus demonstrating the resistance of the probiotic strain to the acid gastric compartment and to bile salts presence (Maathuis *et al.*, 2010). Furthermore, *B. coagulans* BC³⁰ was detected in the fecal samples of subjects that assumed the probiotic strain, in a human trial (Nyangale *et al.*, 2015).

The aim of the present study was the investigation of the stability of the probiotic strain *B. coagulans* BC³⁰, included in a new functional pasta, during the transit through the GI tract. The presence of the probiotic strain was assessed in the fecal samples of twenty subjects who ingested the functional pasta in a 12-week trial. A parallel group of 19 subjects which consumed a control pasta with no added *B. coagulans* BC³⁰ was analysed. Cultivation and molecular analyses were performed to this aim.

2.2. Materials and Methods

Study design

The study design was a randomized, parallel, 12 week trial. Thirty-nine healthy subjects (16 males and 23 females, aged 29-74 years old, BMI > 25 kg/m²) participated to the study. An exclusion *criterium* was the use of food supplements, i.e. prebiotics and probiotics, and antibiotic use in the previous three months. Recruited subjects were assigned in a random order to one of two treatments: a) a functional pasta obtained from whole wheat flour and supplemented with β -glucans from barley and the probiotic strain *B. coagulans* BC³⁰. Subjects assigned to this treatment were named as “treated” group; b) a control pasta produced with the same technological process and from the same whole wheat flour, but without addition of β -glucans and the probiotic strain. Subjects assigned to this treatment were named as “control” group. Detailed characteristics of the functional and control pasta are reported in Chapter 1. Further information on the clinical trial are reported in Chapter 5.

Control and functional pasta

The functional pasta, and in particular the cooked product, contained a microbial load above 6 log CFU/g of the probiotic strain *B. coagulans* BC³⁰, as previously reported (Chapter 1). Instead, the cooked control pasta did not contain bacteria of the genus *Bacillus*.

Collection of fecal samples

At the baseline of the study and every 4 weeks, for a total of 4 times, fresh fecal samples were collected at the Department of Food and Drug (University of Parma), and stored at 4°C in RNAlater (Sigma-Aldrich, Saint Louis, MO, USA). For 5 subjects, fecal samples were collected also few days after the end of the intervention period. For molecular analyses, all samples were further subdivided in 200 mg aliquots and stored at -20°C till processing.

Microbiological analysis on fecal samples

Viable Bacillus spore count

One gram of each fecal sample was mixed with 9 ml sterile peptone (0.1%, w/v) water and mixed with vortex until the sample was thoroughly homogenized. Appropriate dilutions were heat-treated (75°C for 30 min) in a water bath, immediately cooled and pour plated in duplicate in Glucose Yeast Extract (GYE) agar (USP, 2014). Plates were incubated aerobically at 37°C for 48 h, after which the *B. coagulans* colonies were identified on the basis of their characteristic morphology and then counted and recorded. Plate count analyses were performed at the baseline of the intervention period (T0), and after 1, 2 or 3 months (T1, T2 and T3).

DNA extraction procedures

Extraction of DNA from 200 mg fecal sample was performed following the protocol of QIAamp DNA stool Mini Kit (Qiagen, Hilden, Germany), as described by the manufacturer with a lysis temperature of 95°C. DNA concentration and quality were measured using a NanoDrop Lite UV-Vis Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

Quantitative PCR analyses

A quantitative real-time PCR (qPCR) assay was used for the detection of *B. coagulans* BC³⁰ in fecal samples. Enumeration of the probiotic strain was conducted using primers reported in Table 2.1. In particular, these primers, GBI-30F and GBI-30R, were newly designed, basing on comparative analyses on available *B. coagulans* genomes. These primers recognized a region located in a sequence (ID JPSK01000058.1, NCBI) coding for an hypothetical protein, and their specificity was assessed on a collection of *B. coagulans* strains.

Quantitative PCR assays were performed with a LightCycler Nano System (Roche, Mannheim, Germany), using the FastStart DNA Master SYBR Green kit (Roche, Mannheim, Germany), in a final volume of 20 µL, containing 5 µL of each fecal DNA preparation, 0.25 µM of each primer,

MasterMix buffer 1 × and PCR grade water. No-template controls were included in each assay. The thermal cycling conditions used for *B. coagulans* BC³⁰ consisted in one cycle of 95°C for 10 min, followed by 45 cycles with denaturation at 95°C for 15 s, primer annealing at 55°C for 15 seconds, and extension at 72°C for 15 seconds.

A standard curve was constructed by inoculating a pool of fecal samples which did not contained the probiotic strain with 10-fold serial dilutions of freeze-dried *B. coagulans* BC³⁰. Serial dilutions of the inoculated fecal pool were pour plated in GYE agar. BC³⁰ amounts were plotted against the cycle number at which the fluorescence signal increased above the threshold value (C_T value). qPCR amplification efficiency was obtained using the equation $E = 10^{(-1/\text{slope})}$. Cell numbers of bacteria in fecal samples were calculated by comparing the threshold cycle values with the standard curve, and expressed as CFU per gram of feces. All PCR analyses were carried out in technical triplicate, and presented data are the mean values obtained.

Table 2.1. Primer sequences and specifications for qPCR assays (annealing temperature and extension time).

Target	Primer	Sequence (5'-3')	Length (bp)	T _a (°C)	Extension time (sec)	Reference
<i>B. coagulans</i> BC ³⁰	GBI-30F GBI-30R	TCAAAACTCGCATGAAATCG TTCCATCCGAAAGCAAATTC	188	55	15	This study

2.3. Results and discussion

Plate count analyses

Plating analyses were performed on the fecal samples from the group of subjects who ingested the functional pasta enriched with the probiotic strain (treated group), and results were compared with those obtained from the subjects who ingested the pasta without the probiotic strain (control group).

For both groups of samples, a heat treatment step at 75°C for 30 min was carried out in order to select the heat-resistant spore-forming bacteria. This approach represented a valid strategy to focus on the bacterial population of interest.

At the baseline of the study, observations of the plates obtained from both groups of subjects revealed very heterogeneous colony characteristics (Figure 2.1), for morphology (rough or smooth), opacity (matt or gloss), and colour (white, yellow, pink or reddish). Colonies with different morphology and colour were observed under an optical microscope, that revealed

different types of bacilli, short and stubby or long, found singularly or in pairs. These bacteria constituted the aerobic spore-forming microbiota of the subjects, and they probably belong to Bacilli class, that represent one of the two major groups of spore-formers, together with strict anaerobic Clostridia (Hong *et al.*, 2009). In particular, *Bacillus* spores can be found in feces, at relatively low levels, up to 10^4 spores/g (Hong *et al.*, 2009).

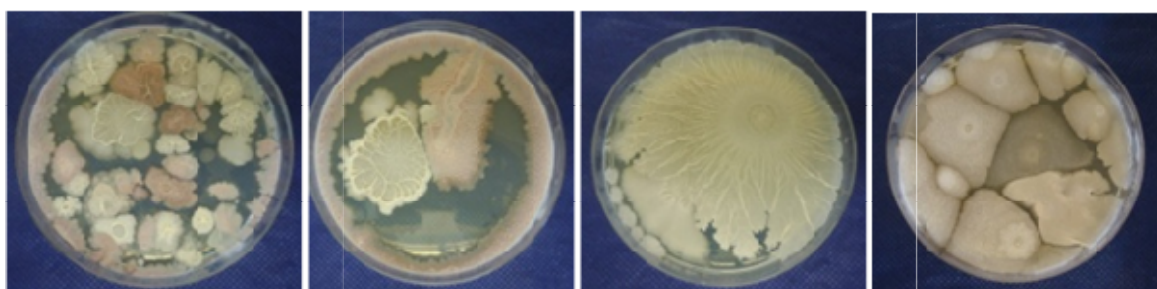


Figure 2.1. Examples of growth observed from fecal samples of the subjects, belonging to the control or treated groups, at the baseline of the intervention period.

For subjects who ingested the control pasta, observed morphologies and colony characteristics continued to be extremely heterogeneous among subjects, during the intervention study. Thus, for the control and treated group at the baseline of the trial, and for the control group, during the trial (T1, T2, T3), the probiotic strain was not detected in the fecal samples. Among the group of subjects who ingested the functional pasta, subjects 2 and 39 represented two exceptions, since they showed the presence of colonies with morphology similar to that of *B. coagulans* BC³⁰ at the baseline of the study, at high amounts (above 10^6 CFU/g).

Plates from the analyses of fecal samples of the treated group, during the treatment period (T1, T2, T3) showed round, smooth, white to cream colonies, characteristics of *B. coagulans* BC³⁰, as shown in Figure 2.2.



Figure 2.2. Examples of growth observed from fecal samples of subjects who ingested the functional pasta (treated group) during the intervention period.

From a quantitative point of view, the amounts of *B. coagulans* in the faecal samples of subjects who consumed the functional pasta varied between 10^5 and 10^7 CFU/g during the whole period of intake of the product, as shown in Figure 2.3. These results showed that the probiotic strain survived to the transit through the GI tract. Moreover, during a constant and prolonged consumption of the functional pasta, *B. coagulans* BC³⁰ become the predominant component of the spore-forming fecal microbiota (Figure 2.2). In fact, it overcome the growth of the highly different colonies observed at the baseline of the intervention (Figure 2.1).

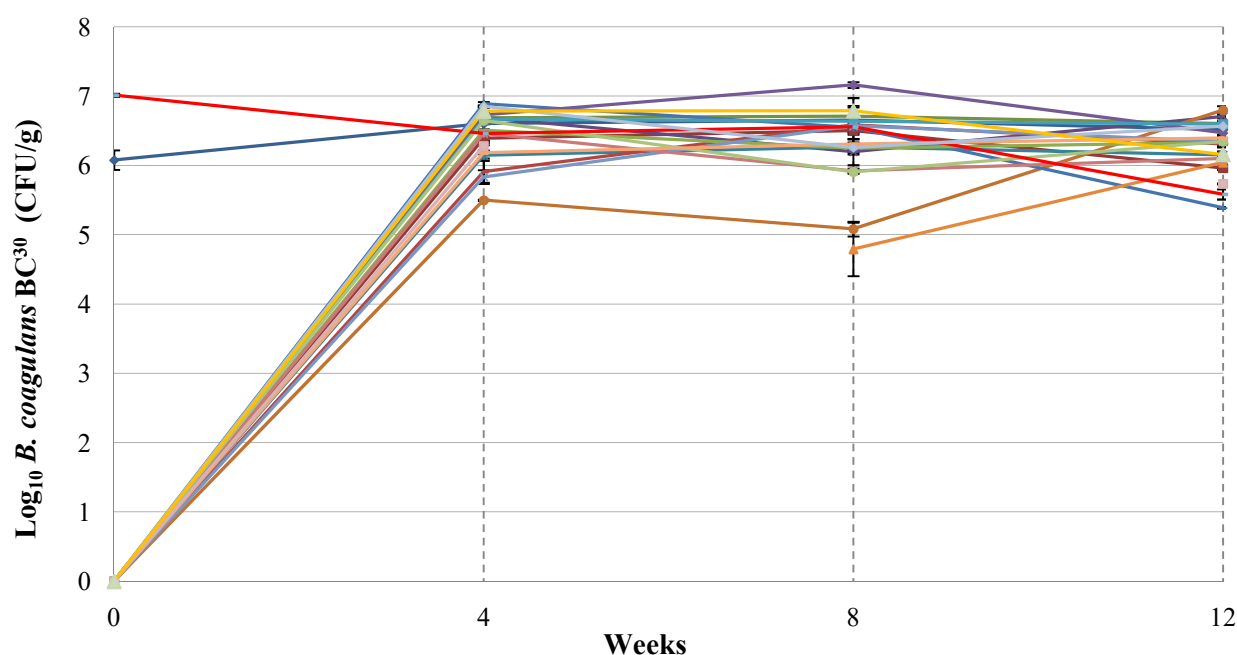


Figure 2.3. Quantification of *B. coagulans* BC³⁰ in the fecal samples of the subjects who ingested the functional pasta, through plate count analysis, before the beginning of the treatment and after 4, 8 and 12 weeks.

qPCR analyses

The study of the persistence of the strain was carried out through quantitative PCR to complete and confirm results obtained from culture-dependent analyses. These analyses were conducted at the baseline (T0) and at the end (T3) of the trial, for the control and treated groups of subjects.

Quantification of the probiotic strain was performed through the use of a standard curve presenting the equation $y = -3.69x + 51.67$, and obtained with the newly designed primers GBI-30F and GBI-30R, that presented an efficiency of 1.87. The limit of detection of the method was 10^3 CFU per assay, which corresponded to 10^5 CFU/g of feces.

Regarding analyses of the control group, the presence of BC³⁰ was not detected at the end of the trial, confirming the fact that these subjects ingested a product that did not contain the probiotic strain. The analyses of the samples of the treated group at time 0 confirmed that BC³⁰ was not

present in the fecal samples, except for the two subjects (2 and 39) who also showed the presence of the probiotic strain at the baseline of the intervention period, through plate count analyses. These two subjects reported later that they ingested the functional pasta few days before the baseline time (T0). Analyses of samples of the treated group, at time 3, confirmed that the probiotic strain was found at high concentration at the end of the clinical trial, in the range of 10^5 - 10^8 CFU/g of fecal sample.

Comparing results obtained from plate count and qPCR analyses it was possible to observe that values obtained with qPCR analyses were generally higher than those obtained from plate analyses (Figure 2.4).

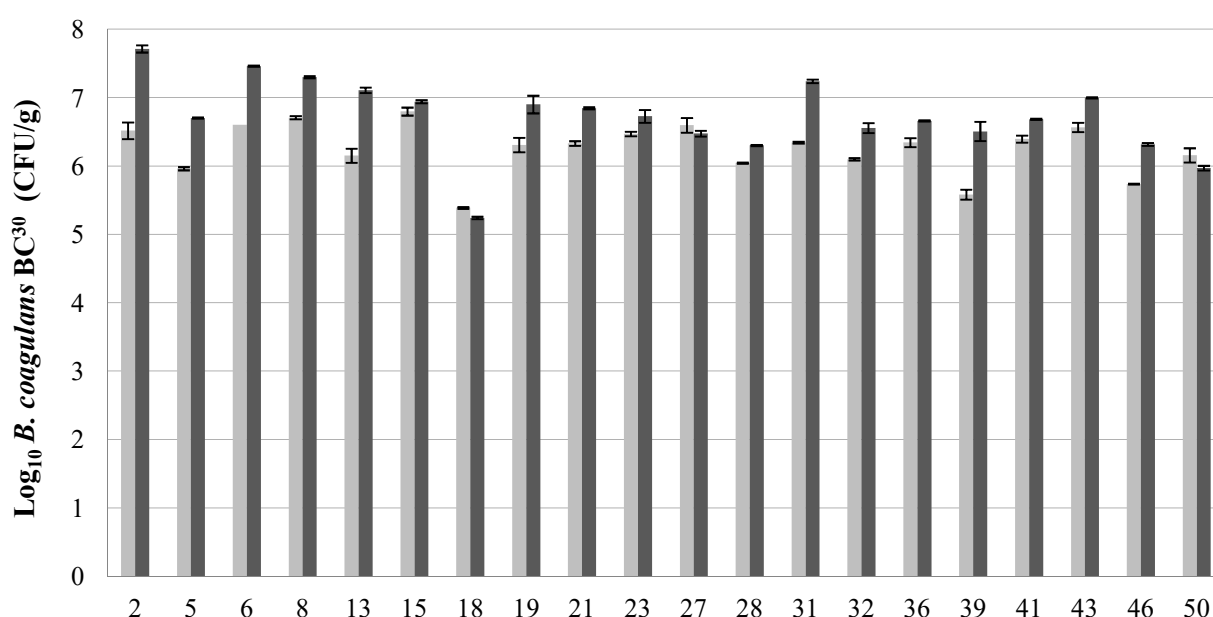


Figure 2.4. Quantification of *B. coagulans* BC³⁰ in the fecal samples of the subjects who ingested the functional pasta, through plate count analysis (in light grey) and qPCR (in dark grey), at the end of the intervention study (T3).

These results were in agreement with previous evidences from literature dealing with quantification of probiotics in fecal samples; in these studies it was reported that molecular methods (e.g. qPCR) led to quantification values one or two orders of magnitude greater than those obtained through classical microbiology methods (Ahlroos and Tynkkynen, 2009). The reason of this may be related to the presence in the fecal samples of non-viable or viable but non-culturable cells (VBNC), that could not grow in plate assays, but whose DNA is detected with molecular techniques. In the case of the present study, dead or damaged vegetative cells

following the heat treatment applied to analyse the fecal samples could represent a remarkable quote of non-viable cells that was not revealed in plate count analyses.

Analyses of the follow-up samples

Some days after the end of the period of intake of the functional pasta, fecal samples from few subjects (32, 36, 37, 39 and 46) were analysed to verify the persistence of the strain after the end of the consumption of the product (follow-up). The aim of this part of the work was to estimate BC³⁰ transit time in the GI tract, namely the period in which the strain was found in fecal samples from the end of the consumption period (Hong *et al.*, 2005a).

Once that ingestion of the functional pasta was stopped, plate count analyses revealed the presence of colonies with different morphology than BC³⁰, probably due to the growth of the other spore-forming bacteria. In fact, in the samples of the follow-up, the strain may have been present in lower amounts compared to the previous sampling time, and its growth in the plate could have been inhibited by the presence of these other colonies. Another attempt was performed by incubating the plates in anaerobic conditions, where it was possible to observe the growth of typical colonies of strain BC³⁰. Thus, anaerobiosis inhibited the growth of other spore-forming bacteria, allowing the detection of the probiotic strain, that could grow since it is a facultative anaerobe.

The quantification of the strain BC³⁰ in the samples of the five subjects is represented in Figure 2.5. In particular, samples were collected after 4 days (2 subjects, 36 and 39), 5, 8 and 9 days (one subject for each time, with code 46, 37 and 32, respectively).

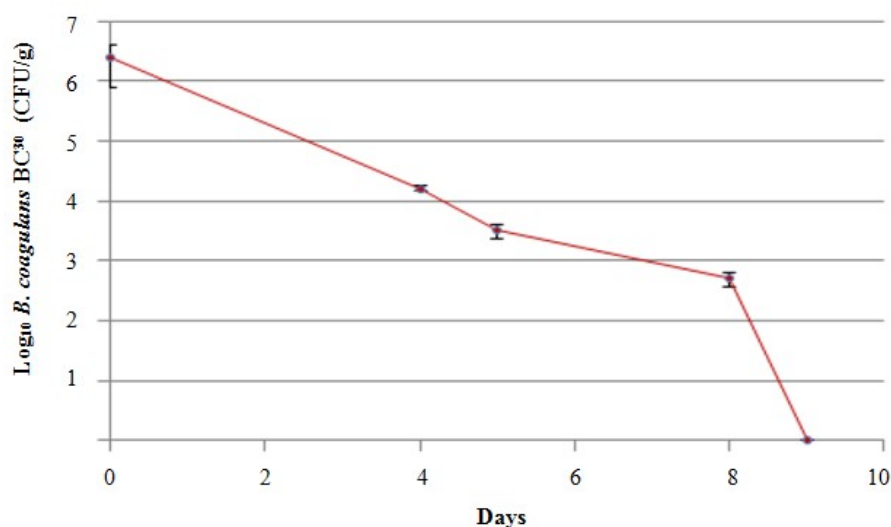


Figure 2.5. Quantification of *B. coagulans* BC³⁰ in the fecal samples of the subjects who ingested the functional pasta, through plate count analysis, during the follow-up period.

BC³⁰ counts decrease from a value above 10⁶ CFU/g (average value of the whole group of subjects who consumed the functional pasta) at the end of the trial, to a value of 10⁴ CFU/g after four days. After five days, BC³⁰ level was reduced by a further logarithmic unit, reaching 10³ CFU/g, and after eight days a further reduction was observed, with levels around 10²-10³ CFU/g. After nine days the strain was no longer found in fecal samples.

The presence of the strain BC³⁰ in the fecal samples of the follow-up period was assessed also through qPCR. Given the low values obtained from plate count analyses, below the limit of detection of the qPCR method (10⁵ CFU/g), DNA isolation was performed not directly from the fecal samples, as it was done for all the other samples, but from the colonies grown on plates. Thus, all colonies were harvested for DNA extraction and qPCR analyses were performed in order to detect the strain, also if present in low proportions and/or even in the case where BC³⁰ growth was partially masked by other colonies. In these assays, a quantification was not performed, but a qualitative information was retrieved, i.e. the presence/absence of the melting peak characteristic of BC³⁰ amplified fragment.

Analyses were done for subjects 36, 37, 39 and 46. Subject 32 was not considered since strain BC³⁰ did not show any growth in plate analyses. The results indicated the presence of the BC³⁰ related melting peak for all samples analysed, excepted subject 37, which showed no amplification products. The fecal sample from this subject was collected eight days after the end of the consumption of the functional pasta, and it provided the lowest count value among the samples of the follow-up.

In conclusion, results from this part of the study showed that the strain BC³⁰ transiently colonised the GI tract of the subjects who consumed the functional pasta, with a residence time of approximately 8 days. In literature, it is reported that probiotic ingested strains are still detected after few days, but rarely after one week (Derrien and van Hylckama Vlieg, 2015). For example, a similar time to that observed for BC³⁰ was reported for *B. stearoothermophilus*, that showed a residence time in the GI tract of 8-10 days (Hong *et al.*, 2005a). On the contrary, in another study, *B. polyfermenticus* was found in fecal samples within 4 weeks from the end of the ingestion (Park *et al.*, 2002). In the first case, the strain simply transited through the GI tract, while in the second study the strain was able to persist in the gut for longer time, probably thanks to specific adhesion properties (Hong *et al.* 2005a).

The dose of orally ingested strains from fermented foods and probiotics ranges between 10⁸ and 10¹² CFU per day (Derrien and van Hylckama Vlieg, 2015). In the present study, for 70-100 g of functional pasta consumed from the subjects, a minimum dose of 10⁸ CFU of *B. coagulans* have

been ingested daily, for 12 weeks. Results from the present study showed that the probiotic strain survived to the transit through the GI tract and was found at high levels in the fecal samples of the subjects, with an average value on the whole group of subjects above 10^6 CFU/g of fecal samples, after one (T1), two (T2) and three (T3) months of functional pasta intake (Figure 2.6). Since plate count analyses were performed after the thermal treatment, in order to select the population of interest in the total fecal microbiota, the fore mentioned values were related to the spore content in the fecal samples. The data demonstrated that the probiotic strain transited intact in the GI tract.

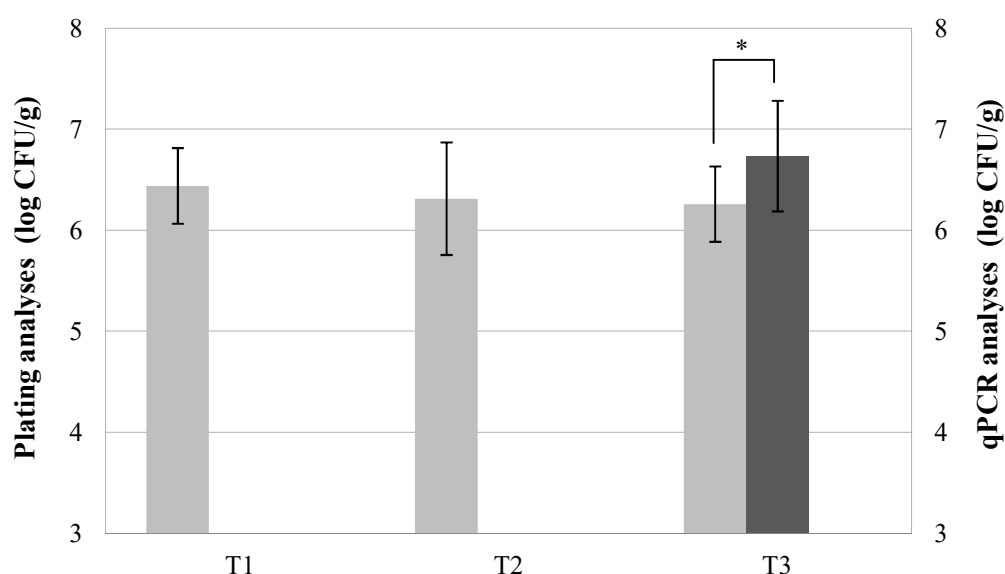


Figure 2.6. Average *B. coagulans* BC³⁰ levels in the fecal samples of the subjects who ingested the functional pasta, through plate count analysis (in light grey) and qPCR (in dark grey). *: $p < 0.05$.

This finding confirmed the stability and resistance of spore-forming probiotics belonging to the genus *Bacillus* (Cutting *et al.* 2011), and reported for the strain *B. coagulans* BC³⁰ from *in vitro* (Maathuis *et al.*, 2010) and *in vivo* (Nyangale *et al.*, 2015) evidences.

However, regarding administration of dormant bacterial probiotics, the discussion is opened about how they can exert a beneficial effect on the gastrointestinal microbiota (Ghelardi *et al.*, 2015). In fact, only following spore germination into vegetative cells a probiotic can exert its effects, e.g. manifesting immunomodulatory properties, secreting antimicrobials or other metabolites (Tam *et al.*, 2006). Studies in animal models showed that *Bacillus* spores, from different *Bacillus* species, e.g. *B. subtilis*, *B. licheniformis*, *B. polyfermenticus*, can germinate in the small intestine, grow, proliferate and then re-sporulate (Casula and Cutting, 2002; Tam *et al.*, 2006; Leser *et al.*, 2008; Jung *et al.*, 2012). Regarding human studies, few information is available. In a recent research, the persistence of a mix of *Bacillus clausii* strains following oral

administration has been evaluated in a human trial (Ghelardi *et al.*, 2015). *Bacillus clausii* is a species used in pharmaceutical preparations for the treatment or prophylaxis of intestinal dysbiosis, that presented several properties e.g. antimicrobial and immunomodulatory activity, involvement in immune responses, cell growth and differentiation, cell adhesion, signal transcription and transduction, secretion of vitamins (Ghelardi *et al.*, 2015). In the study, the authors highlighted that most of these positive functions could only be carried out if the probiotic strain survives, germinates and grows in the gut. The results of the study indicated that the probiotic mix could germinate, since the strains were found in the fecal samples of the subjects after 12 days, after a single dose administration. In fact, considering that the average transit time into the colon is about 2 days (Graff *et al.*, 2001), such a longer transit time would probably have implied the germination of the spores.

With respect to the probiotic strain BC³⁰, an *in vitro* study dealing with the topic of germination of the spores is available in literature (Maathuis *et al.*, 2010). In this research, when a dose of 2×10^9 spores of *B. coagulans* BC³⁰ were introduced into the gastric compartment, the 70% of the spores survived and, moreover, germinated cells were found to be 13.9%. Since in the study the spores were administrated without any growth medium, the authors suggested that, probably, in conditions more similar to those of the GI, germination could have been more pronounced.

Regarding the present study, two evidences could support the hypothesis of the germination of the probiotic strain. First, a statistically significant difference was found between BC³⁰ levels at the end of the trial (T3), observed through plate counts and qPCR analyses (6.3 log CFU/g *versus* 6.7 log CFU/g) (Figure 2.6). If plate count analyses provided a value related to the number of viable spores, results from qPCR reflected the amount of the entire population (spores and vegetative cells), since no thermal treatment was done prior DNA isolation from the fecal samples. Thus, the differences between the two values could be due to the contribution of vegetative cells, derived from germination of the spores and proliferation. Secondly, the spores of *B. coagulans* BC³⁰ were counted after 8 days from the end of the treatment with the functional pasta, so after a period longer than the gastrointestinal transit time, estimated to be from a minimum of 14 hours to a maximum of 32 hours (Derrien and van Hylekama Vlieg, 2015). This could indicate that BC³⁰ could have germinated into the GI tract, according to what reported from Ghelardi and colleagues (2015). Regarding this second point, the evidence obtained from the present study was less robust than the one from the former study, for several reasons. In the study from Ghelardi and colleagues, the mix of *B. clausii* strains was administered in a single dose, while in the present study the subjects assumed the functional pasta continuously for 12-

week; this could physiologically led to a quite long time needed to work off all the spores. Moreover, in the present study, the analyses of the follow-up period were performed on a very small number of subjects. Finally, BC³⁰ amounts found in the follow-up period (10^4 after 4 days) were considerably lower than levels observed during the treatment.

In any case, taking these findings and considerations together, germination of the probiotic strain BC³⁰ could be hypothesised in the investigated subjects, but further studies will be necessary to clarify these aspects.

2.4. Concluding remarks

This study evaluated the survival of the probiotic strain *B. coagulans* BC³⁰ following the transit through the GI tract. In particular, the strain was administered as included in a functional pasta, and the evaluation of its persistence was performed based on classical and molecular analyses of fecal samples of the subjects who participated to the intervention trial.

Results from the study indicated that strain BC³⁰ survived to the transit through the GI tract and its spores were found at a high concentration (above 10^6 CFU/g) in the fecal samples. Thus, the functional pasta investigated in this study represented a good daily vehicle of the probiotic strain, also considering that pasta constitutes a must product of the Mediterranean diet.

The probiotic strain was found to transiently colonize the GI tract of the subjects. In fact, it was found in the fecal samples 8 days after the end of the intervention period with the functional pasta. Consequently, to guarantee high levels of the probiotic strain in the gut, a constant consumption of the functional food is necessary.

Differences in BC³⁰ levels obtained through plate count analyses after a thermal treatment (viable spore count) and qPCR (quantification of spores and vegetative cells) were significant and could be related to a quote of vegetative cells that germinated, grew and proliferated. Moreover, the presence of the probiotic strain in the fecal samples some days after the end of the intervention treatment could represent another evidence supporting the hypothesis of the germination of the probiotic strain in the GI tract. However, given the small number of subjects monitored during the follow up period, further studies will be necessary to clarify the aspect of germination.

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Second part

Chapter III

A case-control study for assessing gut microbiota composition and intestinal permeability in Italian children at risk for type 1 diabetes and healthy children

3.1. Introduction

Type 1 diabetes (T1D), an autoimmune disease caused by immune cell-mediated destruction of insulin-secreting pancreatic beta cells, is preceded by the presence of genetic, immunological and/or metabolic markers, in the absence of clear clinical symptoms. Immunological markers are indicative of pancreatic organ-specific autoimmunity, which can be demonstrated months or years before the clinical onset of T1D (Atkinson *et al.*, 2014).

Evidences from both rodent model (Roesch *et al.*, 2009) and human studies (Vaarala *et al.*, 2008; Bach and Chatenoud, 2012) suggest that the composition of gut microbiota has a significant impact on the immune system development and function. To date, only a few studies explored microbiota composition in children with T1D (Giongo *et al.*, 2011; Murri *et al.*, 2013; Soyucen *et al.*, 2014; Mejía-León *et al.*, 2014) or in subjects with pancreatic organ-specific autoimmunity (de Goffau *et al.*, 2013; Davis-Richardson *et al.*, 2014; Endesfelder *et al.*, 2014; Kostic *et al.*, 2015), therefore evidences for the role of gut microorganisms in the onset and progression of T1D are still limited. Interestingly, most studies agree in reporting a reduced microbial diversity in subjects with overt or pre-T1D than in controls and, remarkably, microbiota composition in T1D or pre-T1D subjects was found different from that of control subjects (de Goffau *et al.*, 2013; Davis-Richardson *et al.*, 2014; Kostic *et al.*, 2015). However, a typical T1D-associated microbiota has not been identified, as relevant microbial genera were different among studies, possibly due to the different geographical origin of subjects (Kemppainen *et al.*, 2015).

A recent study (Brown *et al.*, 2011), based on whole metagenome sequencing, depicted a functional model to explain the relationship between intestinal dysbiosis and increased permeability (commonly called *leaky gut*). In this model, the fate of lactate is crucial in determining gut health: the presence of bacteria able to convert lactate to butyrate results in increased mucin synthesis and strengthened tight junctions, that are related to improved gut

integrity. On the contrary, conversion to short chain fatty acids (SCFA) different than butyrate reduces mucin and tight junctions with consequent increase of gut permeability and, eventually, onset of T1D. Those microbial activities have been associated to a small number of bacterial genera, namely *Lactobacillus*, *Bifidobacterium*, *Faecalibacterium*, *Subdoligranulum*, *Alistipes*, *Bacteroides* and *Veillonella*, in order of possible action in the lactate production/consumption chain (Brown *et al.*, 2011).

Pancreatic organ-specific autoimmunity in children at risk for T1D (pre-T1D) is associated with increased intestinal permeability, a condition that precedes the clinical onset of the disease (Bosi *et al.*, 2006). However, the mechanisms originating leaky gut in these subjects are not well known. In pre-T1D subjects, the presence of an aberrant gut microbiota may result in immune dysregulation responsible for the increase of intestinal permeability and destruction of pancreatic beta cells, thus influencing the onset of the pathology, in the so called “perfect storm” (Vaarala *et al.*, 2008).

Therefore, the aim of this study was to test, for the first time in an Italian sample of children, the hypotheses that: *i.*) the gut microbiota composition of children with beta cell autoimmunity at risk for T1D, analysed using PCR-DGGE and 16S rRNA gene sequencing profiling, is different from that of autoantibody negative children, with particular reference to lactate-producing/consuming bacteria; *ii.*) the intestinal permeability of children with beta cell autoimmunity at risk for T1D is higher than in controls; and *iii.*) there is a possible relationship between these two phenomena.

3.2. Methods

Study participants and design

This case-control study comprised 10 children at risk for T1D (Group: Cases), positive for at least one diabetes associated autoantibody (glutamic acid decarboxylase autoantibodies, GADA; insulin autoantibodies, IAA; insulinoma-2-associated autoantibodies, IA-2A), independently of HLA-DQ genotype, and 10 healthy children (Group: Controls). Regarding children at risk for T1D, four have a first degree relative with T1D, while six have been screened for pancreatic autoimmunity and HLA owing to hyperglycemic episodes. Inclusion criteria were age (range: 6-16 years), normal weight (BMI <85th percentile for age and gender, with Italian BMI percentile tables as reference (Cacciari *et al.*, 2006), residence in Veneto region (North East Italy), exclusion criteria were chronic diseases, dieting, eating disorders, overweight or obesity, chronic use of drugs, presence of active infections, use of antibiotics, probiotics, prebiotics or any other

medical treatment influencing intestinal microbiota during the 3 months before the start of the study. The protocol was in accordance with the 1975 Declaration of Helsinki as revised in 2008 and has been approved by the Institutional Ethics Committee of Verona (Italy).

During the days before the tests, no attempt was made to influence the usual diet of each child. A fecal sample from children was collected using stool collection vials and immediately stored in home refrigerator (4°C) until delivered, within 12 hours, to the study centre and stored at -20°C till bacterial DNA isolation.

Gut microbiota analyses

PCR amplification, DGGE analysis and molecular bacterial identification

Polymerase Chain Reaction followed by Denaturing Gradient Gel Electrophoresis (PCR-DGGE) consists of a PCR amplification of total DNA isolated from a specific matrix (e.g., stool samples) and a subsequent separation of the obtained mixture of amplicons in a denaturing gradient; this results in a profile, where bands are resolved on the basis of their melting temperature/sequence, which can be identified through band sequencing (Muyzer *et al.*, 1993).

Isolation of total DNA from stool samples was carried out using the QIAamp® DNA Stool Kit (Qiagen, Venlo, The Netherlands). PCR fragments of 200 bp representing total fecal bacteria were amplified with the universal primers HDA1-GC and HDA2 as reported before (Walter *et al.*, 2000) (Table 3.1). Moreover, specific bacterial genera (*Lactobacillus*, *Bifidobacterium*, *Bacteroides-Prevotella-Alistipes*, *Clostridium*, *Faecalibacterium* and *Veillonella*) were targeted using primer sets with a reduced amplification specificity (Table 3.1).

Reaction conditions used were identical to those for HDA1-GC and HDA2, except for the annealing temperature (Table 3.1) and longer amplicons obtained were used as templates for nested PCRs performed with the universal primers. DGGE analysis of PCR amplicons was carried out using the Dcode Universal Mutation Detection System (Bio-Rad, Richmond, CA, USA) as described previously (Marzotto *et al.*, 2006). The linear denaturing gradients of urea and formamide used for separation of amplicons are reported in Table 3.1.

DGGE patterns were analysed with the software package UVI band Map (UVItec, Cambridge, United Kingdom). Similarities between DGGE profiles were determined using the Dice coefficient and the unweighted-pair group method with the arithmetic average (UPGMA) clustering algorithm. The number of bands of each individual in every DGGE profile was considered as an indicator of diversity of the fecal microbiota.

Table 3.1. Sets of primers used in this study with the corresponding annealing temperature in PCR reaction, and gradients used for the DGGE gels.

Target	Primer	Sequence	Lenght (bp)	T _a (°C)	Gradient	Reference
Total Microbiota	HDA1-GC HDA2	ACTCCTACGGGAGGCAGCAGT-(GC) GTATTACCGCGGCTGCTGGCAC	200	56	30-60%	Walter <i>et al.</i> , 2000 Walter <i>et al.</i> , 2000
<i>Bacteroides</i> - <i>Prevotella</i> - <i>Alistipes</i>	Bac303F Bac708R	GAAGGTCCCCACATTG CAATCGGAGTTCTTCGTG	418	58	30-55%	Bernhard and Field, 2000 Bernhard and Field, 2000
<i>Bifidobacterium</i>	Bif164mod Bif662	GGGTGGTAATACCGGATG CCACCGTTACACCGGGAA	520	58	45-60%	Satokari <i>et al.</i> , 2001 Satokari <i>et al.</i> , 2001
<i>Clostridium</i> cluster XI	HDA1-mod CXI-R2	ACTCCTACGGGAGGCAG GAGCCGTAGCCTTTCAC	280	58	30-60%	This study Song <i>et al.</i> , 2004
<i>Clostridium</i> cluster XIV	HDA1-mod CXIV-R2	ACTCCTACGGGAGGCAG CTACGCWCCCTTTACAC	250	48	30-60%	This study Song <i>et al.</i> , 2004
<i>Faecalibacterium</i>	HDA1 Fprau645R	ACTCCTACGGGAGGCAGCAGT AATCCGCCTACCTCTGCACT	315	64	30-60%	Walter <i>et al.</i> , 2000 Ramirez-Farias <i>et al.</i> , 2009
<i>Lactobacillus</i>	Lac1 Lac2	AGCAGTAGGGAATCTTCCA ATTYCACCGCTACACATG	340	61	30-55%	Walter <i>et al.</i> , 2001 Walter <i>et al.</i> , 2001
<i>Veillonella</i>	Vpar-longF HDA2	ATCAACCTGCCCTTCAGAGGG GTATTACCGCGGCTGCTGGCAC	390	64	30-60%	This study Walter <i>et al.</i> , 2001

Selected unknown DGGE bands, present in more than 50% of the subjects in one of the two groups, were excised from the denaturing gels, re-amplified with the universal primers without the GC-clamp and cloned with the cloning kit pGEMT-easy vector system (Promega, Madison, WI, USA). Recombinant plasmids were sequenced at the GATC biotech centre (Koln, Germany). Analyses to determine the closest relatives of the partial 16S rRNA gene sequences retrieved were conducted in EzTaxon database (Kim *et al.*, 2012).

16S rRNA gene profiling

Genomic DNA isolation from samples was done using standard molecular biology kits from ZYMO Research provided by Baseclear (Leiden, the Netherlands). PCR amplification of the 16S rRNA gene (V3-V4 region), barcoding and library preparation were performed by BaseClear. Short paired-end sequence reads were generated using the Illumina MiSeq system and converted

into FASTQ files using the BCL2FASTQ pipeline version 1.8.3. Quality trimming was applied based on Phred quality scores. Subsequently sequences were analyzed using the QIIME-pipeline. The dendrogram was constructed by calculating the Bray-Curtis distance between each sample, after which neighbor joining was used for clustering. For comparative analyses of the samples at the species level, the threshold of 0.1% on the total composition was fixed, i.e species found at lower values were not considered.

Intestinal permeability test

Gut permeability was investigated with lactulose/mannitol test (LA/MA test). This consists in the administration of an oral dose of two probes, lactulose and mannitol, and in the measurement of their intestinal uptake by evaluating their urinary excretion in the following 5 hours. The amount of sugars was administered according to the age of children. After an overnight fast and collection of a basal urine sample, children ingested 100 ml of water containing 5 g lactulose and 2.5 g mannitol (double doses for children over 10 years). Urine were collected during the next 5 hours in a container, with the addition of chlorhexidine as a preservative. At the end of the test the total volume of urine was measured and a 10 ml sample was stored at -20°C until the assay. Lactulose and mannitol concentrations in urine were measured using a HPLC method (Marsilio *et al.*, 1998). Results were expressed as a percentage of the administered lactulose and mannitol excreted in urine and as a lactulose:mannitol ratio (LMR).

Biochemical analyses

HbA1c was measured using high-performance liquid chromatography with variant II cation exchange column (Bio-Rad, Richmond, CA, USA). HLA-DQA1 and DQB1 genotyping was performed by sequence specific PCR using AllSet+ Gold High-Resolution Kit (Life Technologies, Carlsbad, CA, USA).

Data analysis

All results were shown as mean and standard deviation of the mean (SD). Student *t*-test was used to compare means. Frequency comparisons were performed by χ^2 test. The Spearman's rho correlation coefficient was calculated to estimate correlations between variables (intestinal permeability, HbA1c% and presence of bacterial groups), and these analyses were performed using SPSS 20.0 software for windows (SPSS Inc., Chicago, IL;USA). A p-value <0.05 was considered statistically significant.

Intestinal permeability test, biochemical analyses and data analyses were performed at the Regional Center for Pediatric Diabetes, Department of Surgical Sciences, Dentistry, Gynecology and Pediatrics, University of Verona.

3.3. Results

Gut microbiota analyses

PCR-DGGE analyses

Fecal bacteria were investigated targeting (i) the overall composition with universal primers (Figure 3.1), and (ii) the composition of specific bacterial groups related to lactate metabolism, namely *Lactobacillus*, *Bifidobacterium*, *Bacteroides-Prevotella-Alistipes*, *Clostridium*, *Faecalibacterium* and *Veillonella*.

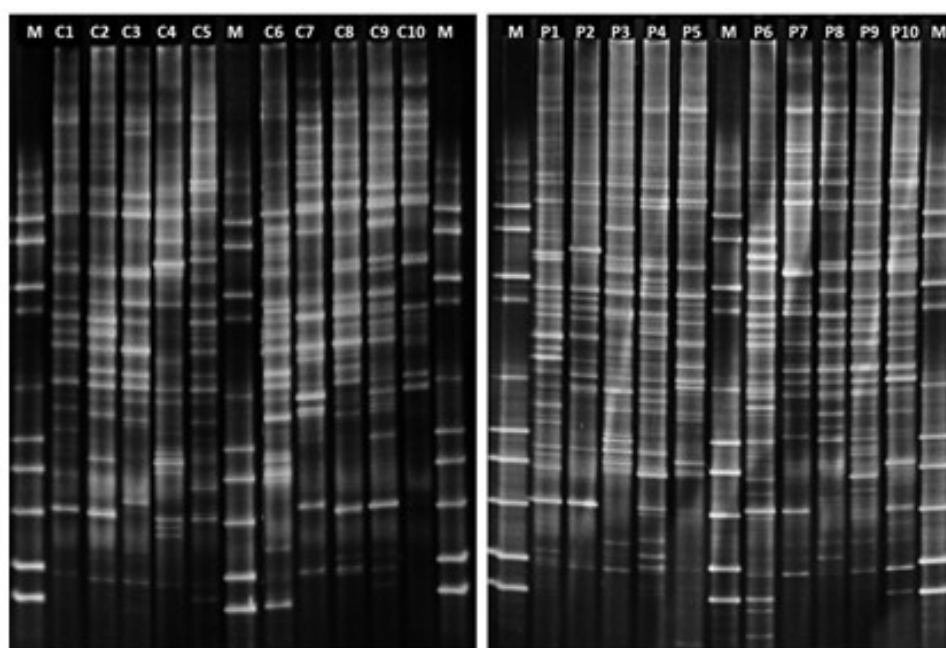


Figure 3.1. Profiles obtained from PCR-DGGE analyses of the total microbiota, for the Controls (C, on the left), and Cases/Pre-diabetics (P, on the right), obtained from the amplification with universal primers HDA1-GC/HDA2.

PCR-DGGE profiles obtained from universal primers displayed very high complexity (average number of bands/profile = 40.2 ± 3.5) and great individual variability but a clear differentiation between Cases and Controls profiles was not possible, as shown in the similarity dendrogram (Figure 3.2) where the two groups are intermixed.

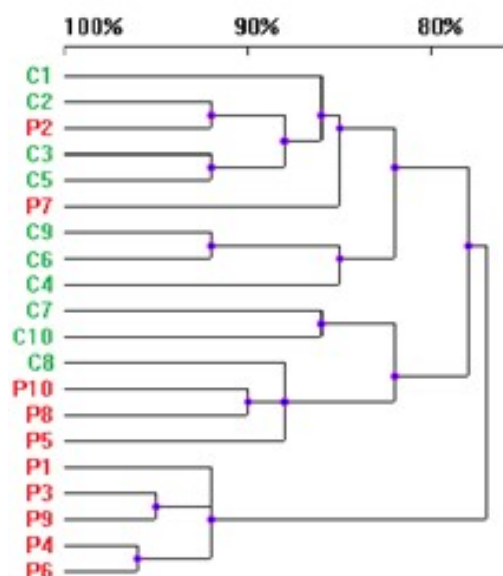


Figure 3.2. Dendrogram obtained from the profiles of the total microbiota of Controls (C) and Cases/Pre-diabetics (P), obtained through PCR-DGGE analyses.

The group-specific DGGE profiles showed a lower complexity than the universal ones (average number of bands/ profile = 11.8 ± 2.5). Again, a clear-cut differentiation between Cases and Controls was not possible. Most representative bands were excised from gels, cloned and sequenced to be identified: 3 from *Lactobacillus*-targeting PCR, 4 from *Bifidobacterium*, 3 from *Bacteroides/Prevotella/Alistipes*, 5 from *Clostridium*, 2 from *Faecalibacterium* and 3 from *Prevotella*, for a total of 20 bands (Table 3).

Table 3.2. Putative bacterial groups identified by sequencing of the DGGE bands amplified from fecal DNA of children at risk for T1D (Cases) and healthy children (Controls), using the group-specific primers. Significant differences between the two groups established at $p < 0.05$ by using the χ^2 test are evidenced in bold.

ID	Closest relative	Identity	Cases (pre-T1D)	Controls	p-value
L1	<i>Gemella sanguinis</i>	98,50%	80% (8)	30% (3)	0,0246
L2	<i>Eubacterium eligens</i>	99,43%	100% (10)	90% (9)	0,3049
L3	<i>Dialister invisus</i>	99,00%	90% (9)	0% (0)	0,0001
B1	<i>Bifidobacterium longum</i> subsp. <i>infantis</i>	99,44%	100% (10)	80% (8)	0,1360
B2	<i>Bifidobacterium longum</i> subsp. <i>longum</i>	98,31%	60% (6)	0% (0)	0,0034
B3	<i>Bifidobacterium</i> sp.	99,45%	70% (7)	40% (4)	0,1775
B4	<i>Bifidobacterium</i> sp.	98,91%	70% (7)	80% (8)	0,6056
Ba1	<i>Bacteroides dorei</i>	99,49%	80% (8)	90% (9)	0,5312
Ba2	<i>Prevotella copri</i>	95,90%	60% (6)	50% (5)	0,6531
Ba3	<i>Bacteroides uniformis</i>	99,49%	80% (8)	80% (8)	1
XI1	<i>Lachnospiraceae</i> sp.	99,43%	80% (8)	40% (4)	0,0679
XI2	<i>Lachnospiraceae</i> sp.	99,43%	90% (9)	90% (9)	1
XI3	<i>Lachnospiraceae</i> sp.	99,43%	100% (10)	100% (10)	1
XIV1	<i>Lachnospiraceae</i> sp.	98,29%	90% (9)	100% (10)	0,2437
XIV2	<i>Barnesiella intestinihominis</i>	99,49%	100% (10)	90% (9)	0,3049
F1	<i>Lactobacillus rogosae</i>	99,43%	100% (10)	100% (10)	1
F2	<i>Eubacterium eligens</i>	98,29%	100% (10)	90% (9)	0,3049
V1	<i>Alistipes shahii</i>	98,97%	70% (7)	60% (6)	0,6392
V2	<i>Veillonella atypica</i>	99,50%	80% (8)	80% (8)	1
V3	<i>Alistipes shahii</i>	98,46%	80% (8)	80% (8)	1

It must be pointed out that sequencing of such a short sequence could not always be considered reliable for identification at the species level, but only at the taxonomic level of the genus. Remarkably, three bands, identified as *Gemella sanguinis* (L1), *Dialister invisus* (L3) and *Bifidobacterium longum* (B2), were present in more than 50% of Cases and only in a few healthy subjects, if any. This could indicate the presence of microbial markers possibly associated to the onset of T1D.

16S rRNA gene profiling

Analyses of fecal microbiota of the children at risk for T1D and healthy children were performed also through 16S rRNA gene sequencing, with the aim of extending the analyses to the overall

fecal communities, thus confirming biomarkers found with PCR-DGGE analyses and possibly expanding the number of candidate biomarkers.

On the overall communities, these analyses confirmed what obtained from similarity analyses of the PCR-DGGE profiles. The two groups, Cases and Controls, resulted intermixed, with no individuation of separated clusters as observed from the dendrogram and PCoA plot (Figure 3.3, Figure 3.4).

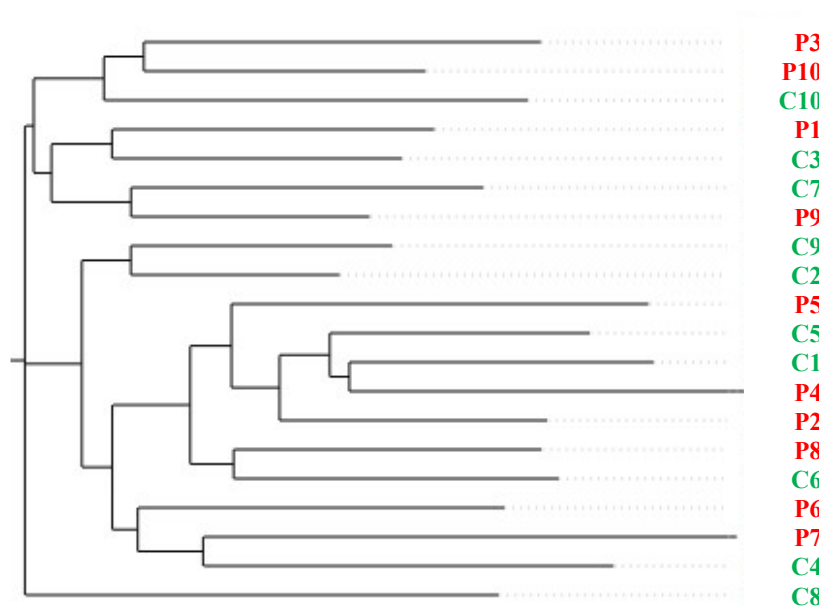


Figure 3.3. Dendrogram obtained from the analyses of the total microbiota of Controls (C) and Cases/Pre-diabetics (P), obtained through 16S rRNA gene profiling.

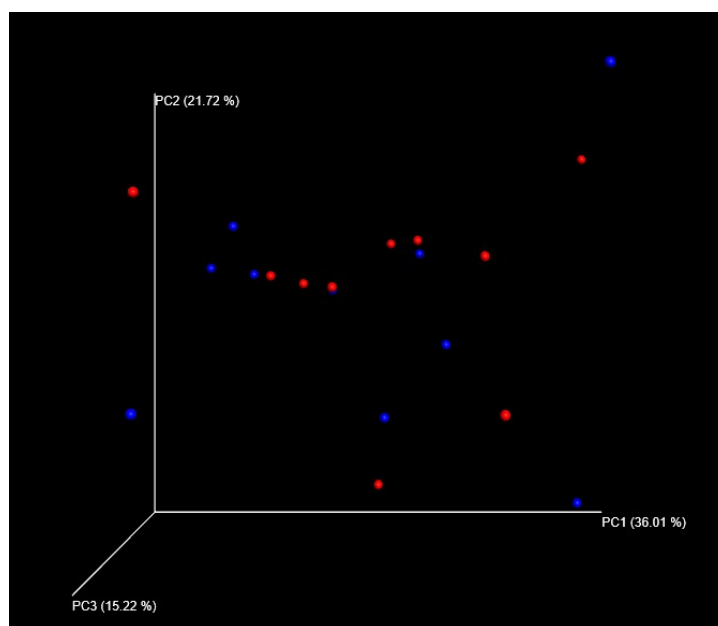


Figure 3.4. Weighted PCoA three-dimensional plot representing microbiota composition of Controls (in red) and Cases (in blue), obtained with UniFrac metric.

A *consensus* list of classified species among Cases and Controls was created, consisting in a total number of 91. The same frequency test applied to the bands found through PCR-DGGE analyses was applied to the species of the *consensus* list. In Table 3.3 species found in the two groups are listed basing on increasing p-value (only species with a difference in the frequencies between the two groups of at least 3 are reported in the table). Moreover, an analysis for comparing abundances in the species of the *consensus* list in the two groups was performed (data not shown).

Table 3.3. Species differentially present in Cases and Controls. Significant differences between the two groups established at $p < 0.05$ by using the χ^2 test are evidenced in bold. Species indicated with ^(a) showed a p-value close to 0.05.

Species	Cases (pre-T1D)	Controls	p-value
<i>Ruminococcus callidus</i>	0% (0)	50% (5)	0.0098
<i>Eubacterium eligens</i>	40% (4)	0% (0)	0.0253
<i>Bifidobacterium bifidum</i>	10% (1)	50% (5)	0.0510 ^(a)
<i>Bifidobacterium pseudocatenulatum</i>	50% (5)	90% (9)	0.0510 ^(a)
<i>Blautia faecis</i>	70% (7)	100% (10)	0.0603
<i>Eubacterium hallii</i>	70% (7)	100% (10)	0.0603
<i>Bacteroides vulgatus</i>	80% (8)	40% (4)	0.0679
<i>Bacteroides ovatus</i>	40% (4)	10% (1)	0.1213
<i>Terrisporobacter glycolicus</i>	10% (1)	40% (4)	0.1213
<i>Clostridium celatum</i>	30% (3)	60% (6)	0.1775
<i>Dialister invisus</i>	70% (7)	40% (4)	0.1775

Two species were found to be differentially present in the two groups, basing on frequencies (presence or absence in the subjects of a group) namely *Ruminococcus callidus* in Controls and *Eubacterium eligens* in Cases. However, these two species were present in a restricted number of subjects in a group (below of equal to 50% of the subjects for each group, Cases and Controls). Thus, these species were not added to previous found markers. Two *Bifidobacterium* species, *B. bifidum* and *B. pseudocatenulatum* were found with a higher frequencies in the Controls. Even if the p-value was slightly above the 0.05 they were not considered as indicative of a certain state (healthy or pre-pathologic), as previous found markers.

No species characterised the Controls or Cases in terms of significantly different abundances.

Regarding biomarkers found with PCR-DGGE analyses, they were not found in association to T1D in the present 16S rRNA sequencing analyses. *Gemella sanguinis* was not found in the samples of pre-diabetic or healthy children, considering the fixed threshold (0.1%). The other two species were found in different frequencies in Cases and Controls (in 7 Cases and 4 Controls for *Dialister invisus* and in 10 Cases and 8 Controls for *Bifidobacterium longum*) than those found with PCR-DGGE.

Intestinal permeability and biochemical measurement

Lactulose urinary excretion results significantly higher in Cases than in Controls (0.313 ± 0.127 vs 0.216 ± 0.072 %; $p=0.043$), while mannitol excretion showed no significant differences between the two groups. The lactulose:mannitol excretion ratio (LMR) was also significantly higher in Cases than in Controls (0.024 ± 0.009 vs 0.017 ± 0.005 ; $p=0.042$), with three subjects above the limit of 0.025, suggestive of altered permeability (Table S3.1). In children with pancreatic autoimmunity we found a significant positive correlation between LMR and HbA1c levels ($\rho=0.718$, $p=0.019$).

Correlation between intestinal permeability and aberrant microbiota

In order to investigate the potential connection between gut integrity and microbiota alteration, the data obtained by PCR-DGGE were compared to those concerning the progression of the disease and the permeability of the gut. Subjects showing the highest number of autoantibodies (Case #1 and #7) were the same with the highest intestinal permeability and almost all of the three microbial markers, whereas those with a lower extent of autoimmunity had a normal intestinal permeability and reduced microbial markers (Case #5 and #10) (Table S3.1).

Moreover, since PCR-DGGE is a semi-quantitative method, the detection of a band is related to the relative abundance of the corresponding microorganism in the sample, at least 1% of the total DNA (Muyzer *et al.*, 1993). Normalisation of DNA concentration in the different samples was performed; therefore we can assume that the relative abundance of the three biomarkers (*Dialister invisus*, *Gemella sanguinis* and *Bifidobacterium longum*) is higher in Cases than in Controls. In this context, a significant correlation between presence of the three microbial markers, in PCR-DGGE, and intestinal permeability, provided by LA/MA test, was found (Figure 3.5) ($\rho=0.542$; $p=0.011$).

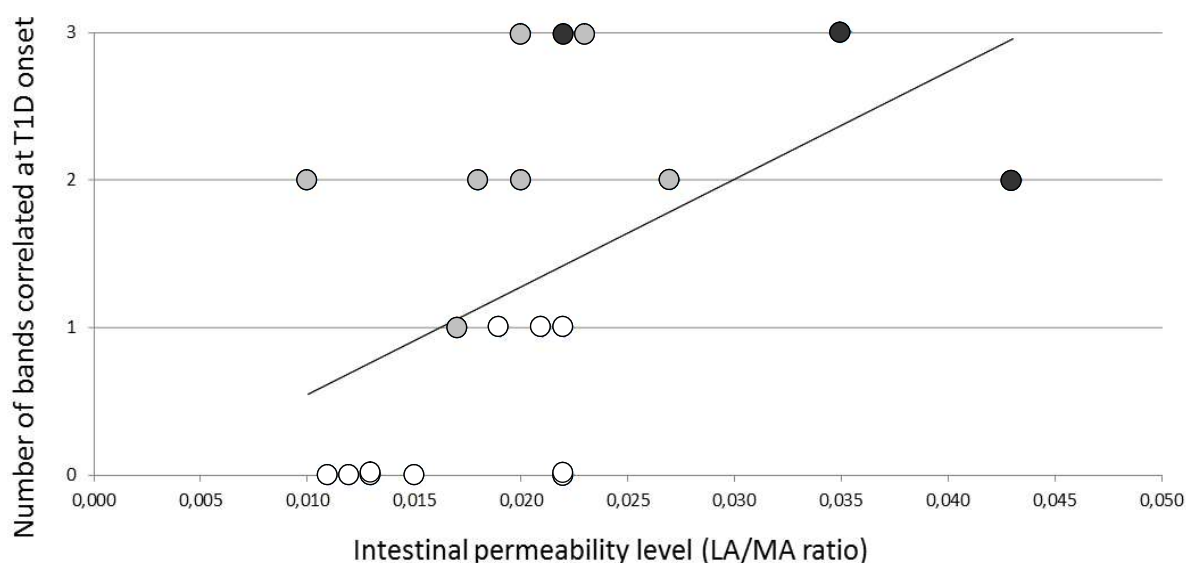


Figure 3.5. Linear correlation between the presence of the microbial markers in PCR-DGGE and intestinal permeability, provided by LA/MA test. White circles = Controls ; grey circles = Cases with 1 or 0 HLA apotype at risk for diabetes and 1 or 2 autoantibodies; black circles = Cases with 2 HLA apotypes at risk for diabetes and at least 2 autoantibodies.

3.4. Discussion

To the best of our knowledge, this is the first study conducted in subjects with pre-T1D in which gut microbiota composition and intestinal permeability were contemporaneously investigated. Moreover, for the first time, Italian children at risk for T1D were investigated for their microbiota composition. The results of this study showed that: *i.*) the gut microbiota composition of pre-T1D children is different, in some components, from that of autoantibody negative children and *ii.*) intestinal permeability of children with beta cell autoimmunity at risk for T1D is higher than in Controls.

Regarding analysis of gut microbiota composition, PCR-DGGE was chosen since it allows a rapid semi-quantitative detection of specific microbial groups, which could be rapidly compared across multiple samples with a low cost and no requirement for specific bioinformatic skills. This technique is one of the most well-established tools for the analysis of complex bacterial communities (Muyzer *et al.*, 1993; Ercolini, 2004; Fraher *et al.*, 2012), it has been recently re-evaluated (Foerster *et al.*, 2014; Ferrocino *et al.*, 2015), and applied also to the analysis of T1D subjects (Murri *et al.*, 2013). In this context, considering the overall composition of the microbiota, our results showed that a clear-cut differentiation between Cases and Controls was not possible; this is in agreement with a previous study reporting analysis with PCR-DGGE

(Murri *et al.*, 2013), and it is probably due to the marked individual variability. However, investigating the microbial populations related to lactate metabolism (lactate-producers/consumers), three species were found statistically associated with beta cell autoimmunity: *Dialister invisus*, *Gemella sanguinis*, and *Bifidobacterium longum*. These markers are different from the species described before as associated to the pre-T1D or T1D group (de Goffau *et al.*, 2013; Davis-Richardson *et al.*, 2014). This could be due to the different techniques applied but, also, to environmental factors, such as differences in geographical origin, eating habits, age or degree of disease progression (i.e. presence of genetic, immunologic or metabolic markers) (Kemppainen *et al.*, 2015). Indeed, literature data refer mainly to Finnish subjects, with a very high genetic risk and aged 0 to 2 years. The impact of such environmental factors on the gut microbiota makes it extremely difficult to recognise common characteristics, universally valid for the description of a pathologic state (Endesfelder *et al.*, 2014). The identification of potential biomarkers appears possible only within a sufficiently homogeneous population (e.g., in terms of geographical and clinical aspects); thus the three markers we found could be characteristic of our specific Italian group of subjects.

From a biological viewpoint, *Dialister* and *Gemella* belong to the category of the so-called pathobionts, i.e. normal inhabitants of the gut in low concentrations (symbionts) that could determine undesired/pathogenic effects where their concentration increase (Chow *et al.*, 2011; Anders *et al.*, 2013). Moreover, in case of altered intestinal permeability, they could also enter the blood stream, possibly inducing complications. On the contrary, *Bifidobacterium longum* belongs to a genus, *Bifidobacterium*, generally considered beneficial to the health of the host, which includes also numerous probiotic strains (Gomes *et al.*, 2014). However, an increase in the level of this genus has already been reported in association with T1D (Brown *et al.*, 2011), and future investigations on the relevance of this category for T1D development could be important. Interestingly, microorganisms found support the functional model suggested in the literature (Brown *et al.*, 2011) as the main products of their metabolism are the SCFA acetate and propionate; these two metabolites, unlike butyrate, cannot stimulate the biosynthesis of mucin and the assembly of the tight junctions, hypothetically leading to an increase of intestinal permeability and finally promoting the autoimmunity reactions (Brown *et al.*, 2011).

Moreover, 16S rRNA gene profiling analyses were performed on samples from the same subjects, in order to extend the analyses to the overall fecal communities, thus confirming previous find biomarkers and possibly expanding the range of candidate biomarkers. These analyses on one hand confirmed the fact that there was not a clear differentiation in the profiles

of Cases and Control, considering the overall composition of the microbiota. On the other hand, at the species level, the three biomarkers individuated through PCR-DGGE were not confirmed. The choice of the analysis technique could have an impact on results, and moreover, any bias introduced in the technical pipeline can potentially contribute to relevant differences (Thomas *et al.*, 2015). In this specific case, several points could be responsible of the different findings obtained with the two techniques about the three microbial species. First, DNA for PCR-DGGE and for 16S sequencing was extracted from two different aliquots of a same fecal sample, and, moreover, using different isolation kits. Also, it is known from literature that different primers for 16S rRNA gene could result in a different richness of amplified species, based on targeted hypervariable regions (Huse *et al.*, 2012). In the case of the present study, the V2-V3 region was amplified for PCR-DGGE analyses while the V3-V4 region was amplified in 16S profiling. Moreover, another important point is that in PCR-DGGE analyses a first PCR step was performed with group specific primers, that selected a certain population, further amplified with universal primers; this procedure could have impacted the proportion of the species in the samples, that eventually resulted different to those observed in 16S profiling. In conclusion, 16S profiling analyses did not reveal any new biomarkers, so *G. sanguinis*, *D. invisus* and *B. longum* remained the species which characterised the pre-diabetic microbiota.

The second main finding of the study, i.e. intestinal permeability of children with beta cell autoimmunity at risk for T1D is higher than in Controls, is in agreement with previous studies conducted in animal models and in individuals affected by T1D as well as in subjects with pre-T1D. Signs of enteropathy were shown to be present in BioBreeding diabetes-prone rats soon after weaning and preceding insulinitis, suggesting that increased intestinal permeability may be necessary for the development of pancreatic autoimmune response (Graham *et al.*, 2004; Neu *et al.*, 2005). This hypothesis was supported also in humans, where intestinal permeability, along with structural mucosal alteration, was found significantly higher in non-celiac subjects with T1D (Secondulfo *et al.*, 2004). Finally, Bosi and co-workers (2006) showed an increased permeability to lactulose not only in long term or new onset T1D but also in first-degree relatives of T1D patients positives for two or more autoantibodies, demonstrating that an intestinal damage precedes the clinical onset of T1D. Nevertheless, it was not clear if autoimmune reaction could develop as a consequence of increased permeability to abnormally adsorbed dietary antigens or, rather, mucosal integrity could be compromised as an effect of inflammation associated to an immune mediated process.

Considering microbial species found and observed increased permeability in pre-T1D children, we evaluated the possible association between gut microbiota and gut integrity, comparing microbiological results with the clinical data related to the intestinal permeability. A significant correlation between the relative abundance of the three microbial markers, evaluated by PCR-DGGE, and the increased intestinal permeability in children at risk of developing T1D was observed (Figure 3.5) ($\rho=0,542$; $p=0,011$).

In our study, also children with positivity to just one autoantibody, suggestive of a lower risk for diabetes onset, were included. Moreover, it is interesting to note that subjects showing the highest number of autoantibodies (Case #1 and #7) were the same with the highest intestinal permeability and almost all of the three microbial markers, whereas those with a lower extent of autoimmunity had a normal intestinal permeability and reduced microbial markers (Figure 3.5). Interestingly, the former had T1D onset a few months after participating in this study.

These preliminary findings seem to support the hypothesis that increased permeability parallels the autoimmune process rather than being a consequence of the metabolic disorder, and that gut microbiota could have a certain influence in the development of the pathological state. Nevertheless, the exact role of gut bacteria in the development of the pathological state remains to be defined, since gut microbiota impairment could be either the cause or a consequence of leaky gut.

The potential strengths of this study are: *i.*) all children participating in the study were living in the same area (Veneto region, North East Italy), eliminating the potential impact of geographic differences on microbiota composition; *ii.*) all children had their intestinal permeability and microbiota composition contemporaneously assessed.

The study has also some potential limitations: *i.*) the sample size is relatively small. This is explained by the fact that only 25-30% of children, first degree relatives of patients with T1D followed in the Center for Pediatric Diabetes, accept to participate in screening programs to identify at risk subjects. The main reason is that, up to now, there are not effective therapies for stopping the autoimmune process leading to T1D and this discourages families to participate in the screening program. *ii.*) PCR-DGGE selected for microbial analyses, even though advantages like being inexpensive, its repeatability, the easy interpretation of the results, the ability to highlight the predominant species present in the microbial community and the ability to modulate the depth of the analysis by carefully selecting the primers, according to their specificity (Fraher *et al.*, 2012), has a limited degree of discrimination at the specie level. However, in the specific case of this study, differences in microbiota components individuated

with PCR-DGGE were not confirmed using a most powerful analyses tool, as 16S rRNA gene profiling. These divergent results, obtained analyzing the same samples, underlined the need of applying more uniform protocols and approaches, also to allow the comparison of results from different studies.

3.5. Concluding remarks

In conclusion, Italian children with beta cell autoimmunity at risk for T1D have a different intestinal microbiota composition and a higher intestinal permeability than healthy children. Taking into consideration the evidence of microbiota variability among populations, an external validity of the results of this study is possible. Future studies are necessary to assess the potential effect of microbiota modifications on the process leading to increased permeability and/or autoimmune mechanisms promoting diabetes onset in order to develop possible therapeutic treatments.

On the basis of the evidences that several disorders are linked to the intestinal microbiota, suppression of dysbiosis could represent an important clinical goal. Even though much fundamental research remains to be done, several promising potential therapeutic options have been proposed (Walker and Lawley, 2013). In particular for T1D, an intervention on the microbiota in the pre-pathological phase has the ambitious goal of delaying the diabetes onset or, at least, of improving the intestinal conditions of the subjects. The use of specific antibiotics or the application of a fecal transplant are two of the principal methods used to restore a healthy microbiota. Therefore, the most applicable method seems to be the modulation of the diet, also introducing a probiotic. In fact, probiotics are single (or defined mixtures of) microorganisms which probably exert their beneficial action through mechanisms of interaction/inhibition with gut microbiota, as well as stimulating the immune response (Corr *et al.*, 2009; Reid *et al.*, 2011). Several studies are already available on this topic (Bron *et al.*, 2011) and the results on the actual effectiveness of probiotics on the treatment of T1D seem promising (Gomes *et al.*, 2014; Dolpady *et al.*, 2016). A better knowledge of gut microbial composition associated with the development of T1D is necessary to be able to choose the best treatment.

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Supplementary material

Table S3.1. Summary of Cases characteristics: HLA genotypes, autoantibodies positivity, HbA1c%, lactulose:mannitol excretion ratio (LMR), and eventual T1D onset. HLA alleles associated with increased risk to T1D are in bold, whereas in italic are showed alleles with decreased risk. Values considered as pathological for LMR and calprotectin are in bold. Asterisk in Case 8 HbA1c indicates that value is not fully informative due to glucose-6-phosphate dehydrogenase deficiency.

Cases (pre-T1D)	T1D first- degree relatives	HLA DQA1-DQB1 (A1B1/A1B1)	Autoantibody positive	HbA1c % (mmol/mol)	LMR	T1D diagnosis (days after test)
1	Yes	0301-0302 / 0501-0201	GAD, IA2, IAA	6,0 (42)	0,035	64
2	No	0301-0302 / 0501-0201	GAD, IA2	5,4 (36)	0,022	-
3	No	0102-0604 / 0201-0202	GAD	5,3 (34)	0,020	-
4	No	0501-0201 / 0501-0201	GAD	5,8 (40)	0,023	-
5	No	<i>0303-0301</i> / 0303-0302	IAA	5,6 (38)	0,010	-
6	Yes	0201-0202 / 03011-0302	IAA	5,8 (40)	0,027	-
7	Yes	0301-0302 / 0501-0201	GAD, IA2, IAA	6,3 (45)	0,043	181
8	No	0401-0402 / 0501-0201	GAD, IA2	5,4 (36)*	0,017	15
9	Yes	0201-0202 / 0201-0303	GAD	5,3 (34)	0,020	-
10	No	0101-0501 / 0301-0302	IAA	5,2 (33)	0,018	-

Chapter IV

An *in vitro* study to investigate the effects of a new functional pasta and its ingredients on gut microbiota from children with different susceptibility to type 1 diabetes

4.1. Introduction

Diet is one of the key elements involved in the modulation of gut microbial composition (Scott *et al.*, 2013). Diet can be intended at different levels, from dietary patterns (e.g. Western diet versus vegetarian diets), to specific foods (e.g. whole grain products) or certain food constituents (e.g. dietary fibres, fat or proteins (Graf *et al.*, 2015), which have impact on the composition and activity of gut microbiota. Diet has an important role in the maintenance of a healthy condition or in specific disorders or pathologies (Conlon and Bird, 2015). To this aim, the development of functional foods, that include prebiotics or probiotics aimed to improve the health status through the modulation of gut microbiota, is a recent emerging area (Bosscher *et al.*, 2009).

In order to study the effects and properties of a new functional food on gut microbiota, and before the *in humana* trial step, a previous *in vitro* evaluation step could be useful to obtain preliminary information (Williams *et al.*, 2015). The TNO *in vitro* model of the colon (TIM-2) (Minekus *et al.*, 1999) is a system that accurately mimics the conditions in the human large intestine, being maintained under well defined controlled parameters (temperature, pH, anaerobiosis), and it can be inoculated with fecal material from subjects of interest. It represents a powerful tool to study the effects of the administration of different substrates on the gut microbiota, under standardised conditions (Venema *et al.*, 2000).

Analyses of microbiota composition through 16S gene DNA profiling is a common strategy to investigate modulations in microbial communities (Fraher *et al.*, 2012). Moreover, analyses of produced metabolites can give complementary information about the effect of the administration of a given treatment on gut microbiota. In particular, short chain fatty acids (SCFA) derived from undigested carbohydrates that are fermented by the microbiota in the large intestine. SCFA, i.e. acetate, propionate and butyrate, can exert different effects on the host (Den Besten *et al.*, 2013) and, for example, among key functions, they represent a source of energy for the host (Bergman,

1990). In particular, butyrate is considered the energy source for the colonic epithelial cells (Roy *et al.*, 2006) and it is considered a beneficial metabolite thanks to its role in modulating the intestinal barrier and to its anti-inflammatory properties (Pryde *et al.*, 2002; Brown *et al.*, 2011). In a previous study, a novel whole grain functional pasta, enriched in a probiotic bacterium *Bacillus coagulans* GBI-30, 6086 (thereafter BC³⁰) and β -glucans from barley was developed (Fares *et al.*, 2015). The effects of the ingestion of the functional pasta on the gut microbiota is not known, yet. However, some information about the effects of the functional ingredients, *B. coagulans* BC³⁰ and β -glucans, are available. β -glucans are reported to have prebiotic properties, leading to the stimulation of specific bacterial genera (e.g. *Lactobacillus*, *Bifidobacterium*) as reported in *in vitro* studies (Hughes *et al.*, 2008; Kedia *et al.*, 2009) or *in humana* (Mårtensson *et al.*, 2005; Mitsou *et al.*, 2010). On the contrary, the effects of the probiotic strain on gut microbiota has been evaluated in few studies (Honda *et al.*, 2011; Nyangale *et al.*, 2014; Nyangale *et al.*, 2015). Furthermore, also the whole dietary fibre included in the functional pasta could be active in the modulation of resident microbiota (Portune *et al.*, 2017).

Type 1 diabetes (T1D) is an autoimmune disorders that causes the destruction of pancreatic β -cells; in T1D a dysbiosis condition has been reported in association to the development of the pathology. Differences in microbiota composition among children with the pathology and healthy children were reported in several case-control studies (Giongo *et al.*, 2011; Murri *et al.*, 2013), and, moreover, differences were reported also for children in the condition of pre-diabetes (de Goffau *et al.*, 2013; Davis-Richardson *et al.*, 2014; Maffei *et al.*, 2016). Regarding the use of intervention studies aimed at modulating gut microbiota in T1D subjects, few studies are available. In particular, the role of probiotics in T1D condition has been studied in animal models (Gomes *et al.*, 2014), but not *in humana*. Moreover, no data about the use of prebiotics for the modulation of gut microbiota in children with T1D is available, but a study protocol has been recently published to assess the effect of prebiotic intake on gut microbiota, intestinal permeability and glycemic control in children with T1D (Ho *et al.*, 2016).

Here, an *in vitro* study with the TIM-2 model was carried out to evaluate the effects of the new functional pasta and its functional ingredients on the gut microbiota coming from children with different susceptibility to T1D, i.e. healthy, pre-diabetic and diabetic children. The functional ingredients investigated were the probiotic strain *B. coagulans* BC³⁰ and β -glucans, tested separately or in combination; then, the functional pasta containing both the functional ingredients, the pasta enriched with only *B. coagulans* BC³⁰, and a control pasta without the two considered ingredients were examined. The investigation of gut microbiota was performed

through 16S rRNA gene profiling for determining composition, and gas chromatography for determining short chain fatty acid (SCFA) production, respectively. In particular, to reveal relationship between dietary interventions and gut microbiota modulation, bacterial diversity within and among samples, and inference of the metabolic capabilities associated with the bacterial microbiome of the three groups of children following each of the tested treatments were investigated. Finally, results from microbiota composition analyses and metabolites were merged.

4.2. Materials and Methods

Intervention meals and SIEM control

A standard ileal efflux medium (SIEM) was used as a control. The composition of this medium was partially modified from previous descriptions (Gibson *et al.*, 1988; Maathuis *et al.*, 2009). Briefly, it contains TBCO [mixture of Tween 80 (33.4 g/L), casein (46.5 g/L), bactopectone (46.5 g/L), and ox bile (0.8 g/L)], CHO [mixture of pectin (5.9 g/L), xylan (5.9 g/L), arabinogalactan (5.9 g/L), amylopectin (5.9 g/L) and starch (49.6 g/L)].

To this standard medium, the specific ingredients of interest were added and in particular: a freeze-dried preparation of *Bacillus coagulans* GBI-30, 6086 (BC³⁰) (Ganeden, Mayfield Heights, OH, USA), in a dose of 10¹⁰ CFU/day, β -glucans (purity > 94%) (Megazyme, Bray, Ireland) in a dose of 4 g/day, the combination of BC³⁰ and β -glucans (10¹⁰ CFU/day and 4 g/day, respectively), a simulated pasta enriched with the probiotic strain, obtained from the control pasta (about 27 g/day) to which the probiotic strain (10¹⁰ CFU/day) was added, the whole grain control pasta (about 27 g/day), the functional pasta (Fares *et al.*, 2015) (about 27 g/day). Pasta samples were previously cooked in water with 1% NaCl added, and subsequently homogenised with a domestic blender. Regarding the meal with the functional pasta, it was diluted with water (50:50) in order to avoid problems due to the high viscosity of the medium, and the same was done also on an aliquot of the meal containing the control pasta. Finally, these two diluted meals included an amount of pasta of about 13.5 g/day.

Microbiota origin and preparation of fecal homogenates

Two healthy children, two children at risk for T1D diabetes and two children with T1D were recruited at the Regional Center for Pediatric Diabetes, Department of Surgical Sciences, Dentistry, Gynecology and Pediatrics, University of Verona. They participated to the previous study described in Chapter 3 (Maffeis *et al.*, 2016), and in particular they represented Controls

C1 and C2, and Cases P1, P3, P6 and P7 of that research. In particular, Cases P1 and P7 manifested T1D onset some months after the study. All children had not used antibiotics, prebiotics, or probiotics for at least 3 months prior to the donation.

Fresh fecal samples were collected by parents in a closed box with an anaerobic strip (AnaeroGen, Oxoid, Cambridge, UK), and delivered to the laboratory where they were snap-frozen in liquid nitrogen and stored at -80°C . After thawing, microbiota for each couple of children (healthy, pre-diabetic and diabetic) was pooled in an anaerobic cabinet. In particular, fecal donations were diluted in a ratio 1:1 in a physiological saline preparation/dialysate (content per litre: 2.5 g $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, 4.5 g NaCl, 0.005 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05 g ox-bile), glycerol was added (10% w/w) as a cryoprotective agent, and this mix was homogenized with a Turrax (IKA Ultra turrax T25 digital, Staufen, Germany). Aliquots of the fecal homogenates were snap-frozen in liquid nitrogen and stored at -80°C till use. Before being introduced into the system, the homogenate was thawed through 1 h immersion in a 37°C water bath.

TIM-2 experiments

The TIM-2 system (Figure 4.1) has been previously described in details (Maathuis *et al.*, 2009). Briefly, the system was kept in anaerobic condition by flushing with nitrogen (Figure 4.1f), maintained at 37°C , monitored by a temperature sensor (Figure 4.1j), and the pH was kept at 5.8 through automatic titration with 2M NaOH (Figure 4.1c). A luminal content of approximately 120 ml was maintained constant and monitored through a level sensor (Figure 4.1e), and peristaltic movements were performed regularly (Figure 4.1a). All parameters were computer-controlled.

A dialysate system consisting of a semi-permeable hollow membrane ran through the lumen, in order to remove water and fermentation products from the lumen (Figure 4.1d). A dialysate solution was flushed through this membrane at a speed of 1.5 ml/min, and it consisted in the physiological saline preparation described in the microbiota section, plus $\text{MgSO}_4 \cdot \text{H}_2\text{O}$ (0.5 g/L), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.45 g/L), cysteine hydrochloride (0.4 g/L), and 0.1% of a vitamine solution containing menadione (1 mg/L), D-biotin (2 mg/L), vitamin B12 (0.5 mg/L), pantothenate (10 mg/L), nicotinamide (5 mg/L), p-aminobenzoic acid (5 mg/L), and thiamine (4 mg/L).

At the beginning of each experiment, about 60 ml of fecal homogenate were inoculated in the system together with 60 ml of dialysate, in order to obtain a final volume of approximately 120 ml. After the inoculation of the microbiota, the previously described SIEM medium was administered to the system for the following 16-hours (adaptation period). After that, the

microbiota was deprived of any medium for 2 hours (starvation) in order to let it consume the substrates received previously. After this starvation period, the meal of interest was administrated to the microbiota, during the following 72 hours.

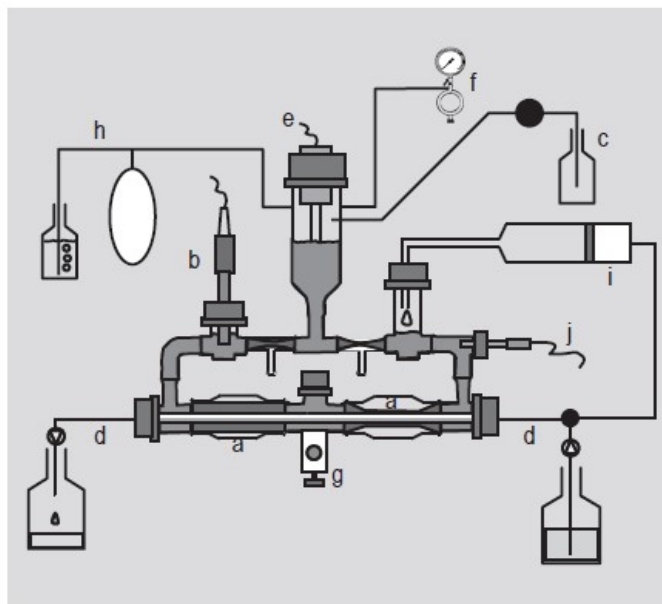


Figure 4.1. Schematic representation of TIM-2 model (from Hatanaka *et al.*, 2012). a) peristaltic compartments containing faecal homogenate, b) pH sensor, c) NaOH secretion, d) dialysate system, e) level sensor, f) nitrogen inlet, g) sampling port, h) gas outlet, i) test compound/SIEM feeding syringe, j) temperature sensor.

The tested meals or the control SIEM medium were gradually introduced into the system through a feeding syringe (Figure 4.1i). In particular 60 ml of the SIEM medium were introduced in the system in the 16-hours adaptation period, while 270 ml of the control SIEM or of a certain tested meal were introduced in the following 72-hours, at a rate of 0.067 ml/min.

Samples from the lumen (Figure 4.1g) and dialysate (Figure 4.1d) were collected at the end of the starvation period (time 0 hours), and after 24, 48 and 72 hours from the beginning of the treatment with the meal of interest (time 24 hours, time 48 hours and time 72 hours, respectively). Samples were analysed for SCFA production at each time point, while they were analysed for microbiota composition at time 0 hours and time 72 hours.

SCFA analyses

SCFA were analysed on a TRACE GC Ultra Gas Chromatograph system coupled with a FID detector (Interscience, Breda, the Netherlands), as described in details by Ladirat and colleagues (2014). Briefly, 50 μ l of samples or standards were mixed with 50 μ l of 0.15 M oxalic acid; after 30 min, 150 μ l water was added and 1 μ l of the sample was analysed.

Microbiota analysis

Genomic DNA isolation from TIM-2 samples was done using standard molecular biology kits from ZYMO Research provided by Baseclear (Leiden, the Netherlands). PCR amplification of the 16S rRNA gene (V3-V4 region), barcoding and library preparation were performed by BaseClear. Short paired-end sequence reads were generated using the Illumina MiSeq system and converted into FASTQ files using the BCL2FASTQ pipeline version 1.8.3. Quality trimming was applied based on Phred quality scores. Subsequently sequences were analyzed using the QIIME-pipeline.

Data analyses and presentation

All experiments were performed in duplicate ($n=2$); in fact, it was previously shown that the number of replicates can be confined to two, since the experiments are highly reproducible, thanks to the fact that conditions are strictly controlled through the computer-connected system, and to the use of a standardized microbiota (Venema *et al.*, 2000). Results are presented as average of duplicates. Regarding microbiota composition, for comparative analyses of the samples at the genus and species levels, thresholds of 1% and 0.1% with respect to the total composition were used, respectively. Modulation was calculated and expressed as the difference between the abundances of a given microbial group, at time 72 hours and time 0 hours (Δ 72h-0h), further normalised for the difference (Δ 72h-0h) of the control meal; in particular Δ 72h-0h of the control meal was subtracted to Δ 72h-0h of the treatment of interest. Regarding SCFA production, the concentration of metabolites at time 0 hour was set to zero. In comparing relative SCFA production (acetate, propionate or butyrate in a same experiment) a difference equal or above 25 mmol was set as a threshold to define the predominance of a metabolite with respect to another. In merging microbiota composition and SCFA production, the increases in SCFA (Δ 72h-0h) were compared with increases in composition (Δ 72h-0h); in this specific case, increases in composition were not further normalised for the control meal.

4.3. Results and discussion

Microbiota composition

Analyses at the beginning of the in vitro interventions

A first investigation was performed on the microbiota from the three groups of children (healthy, pre-diabetic and diabetic children) at the beginning of the *in vitro* experiments (time 0 hours), i.e. after the 16 hour-adaptation period and the 2 hour starvation period in the TIM-2 model.

Analyses were initially performed at the genus level, and expressed, for each of the three groups, as average of the profiles obtained in the replicates (Figure 4.2).

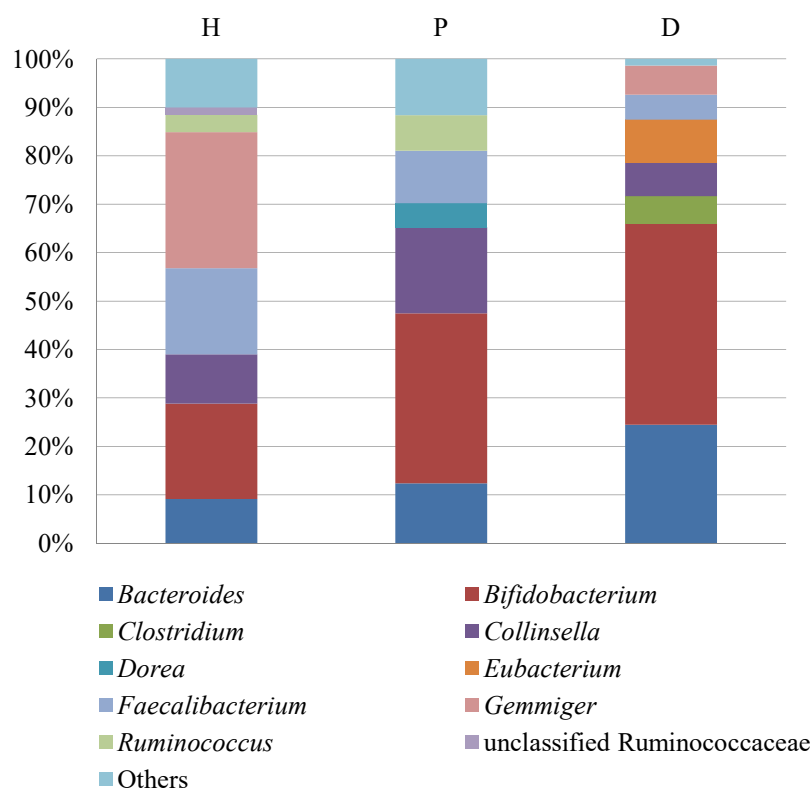


Figure 4.2. Average compositions at the genus level of the microbiota from healthy (H; n=16), pre-diabetic (P; n=15) and diabetic (D; n=18) children at the beginning of the *in vitro* fermentations (time 0 hours).

Results at this time point showed that the microbiota of the three groups of children exhibited differences in the composition, in terms of presence and/or abundances of different genera. *Bacteroides* and *Bifidobacterium* were present in the microbiota from the healthy children (healthy microbiota) at low ratios (9% and 20% of the total microbiota, respectively), and they were higher in microbiota from pre-diabetic children (pre-diabetic microbiota) (12% and 35%) and further more in the microbiota from diabetic children (diabetic microbiota) (24% and 41%), compared to the healthy microbiota. On the contrary, the genus *Gemmiger* was present in high proportion in the healthy microbiota (28%) and in low proportion in the diabetic microbiota (6%). Also *Faecalibacterium* was found at high levels in the healthy microbiota (18%) and it was lower in pre-diabetic (11%) and diabetic microbiota (5%). Differences in average abundances of the genus *Collinsella* were also observed (10% in the healthy microbiota, 18% in the pre-diabetic microbiota, 7% in the diabetic microbiota). Moreover, *Ruminococcus* was

present in healthy and pre-diabetic microbiota (4% and 7%), *Dorea* in pre-diabetic microbiota (5%) and *Clostridium* (6%) and *Eubacterium* (9%) in the diabetic microbiota.

At the species level, different microorganisms appeared to be statistically associated with one of the microbiota from the three children groups. Among these, four microorganisms were markedly associated to the healthy or pre-diabetic or diabetic microbiota. In particular, *Gemmiger formicilis* and *Faecalibacterium prausnitzii* resulted associated to the healthy microbiota, while *Bacteroides vulgatus* and *Bifidobacterium adolescentis* appeared characteristics of both the pre-diabetic and diabetic microbiota. *Faecalibacterium prausnitzii* is notably recognized as a microorganism associated with a healthy condition (Miquel *et al.*, 2014); moreover, both *G. formicilis* and *F. prausnitzii* are butyrate producers (Gossling and Moore, 1975; Duncan *et al.*, 2002); thus, the fact that these two species, that present beneficial characteristics, were found in the microbiota from healthy children, was in agreement with what expected. *Bacteroides vulgatus*, that resulted at higher levels in pre-diabetic and diabetic microbiota, was already reported as associated to pathologic conditions (Finegold *et al.*, 2010). Interestingly, also *B. adolescentis*, belonging to a genus traditionally considered as beneficial, was found at high levels in pre-diabetic and diabetic microbiota. However, *B. adolescentis* was reported as associated to other diseases, such as allergies, IBS, asthma (Sánchez *et al.*, 2010; Arboleya *et al.*, 2016).

Analyses after the 72 h in vitro interventions

Firstly, modulations due to the tested treatments were analysed in each of the three microbiota (healthy, pre-diabetic and diabetic), after normalisation with respect to the effects observed for the control meal.

In the healthy microbiota, eighteen genera resulted modulated after the interventions with the different treatments (Figure 4.3).

Different bacterial groups were decreased, compared to the SIEM control, after treatment with every intervention tested. In particular, *Bacteroides*, *Collinsella* and *Gemmiger* showed marked decreases, while *Dialister* and *Oscillospira* exhibited a decrease of less than 5%.

In the perspective of unravelling a direct relationship diet-microbiota, the focus was mainly on bacterial groups that resulted increased after treatment with a specific diet. Thus, 13 genera were individuated as positively modulated in the healthy microbiota, which are reported in Table 4.1a.

Most remarkable modulations (increases above 5%) were related to the genera *Bifidobacterium*, *Blautia*, *Eubacterium*, *Faecalibacterium*, *Fusicatenibacter*, *Lactobacillus*, *Prevotella* and *Ruminococcus*.

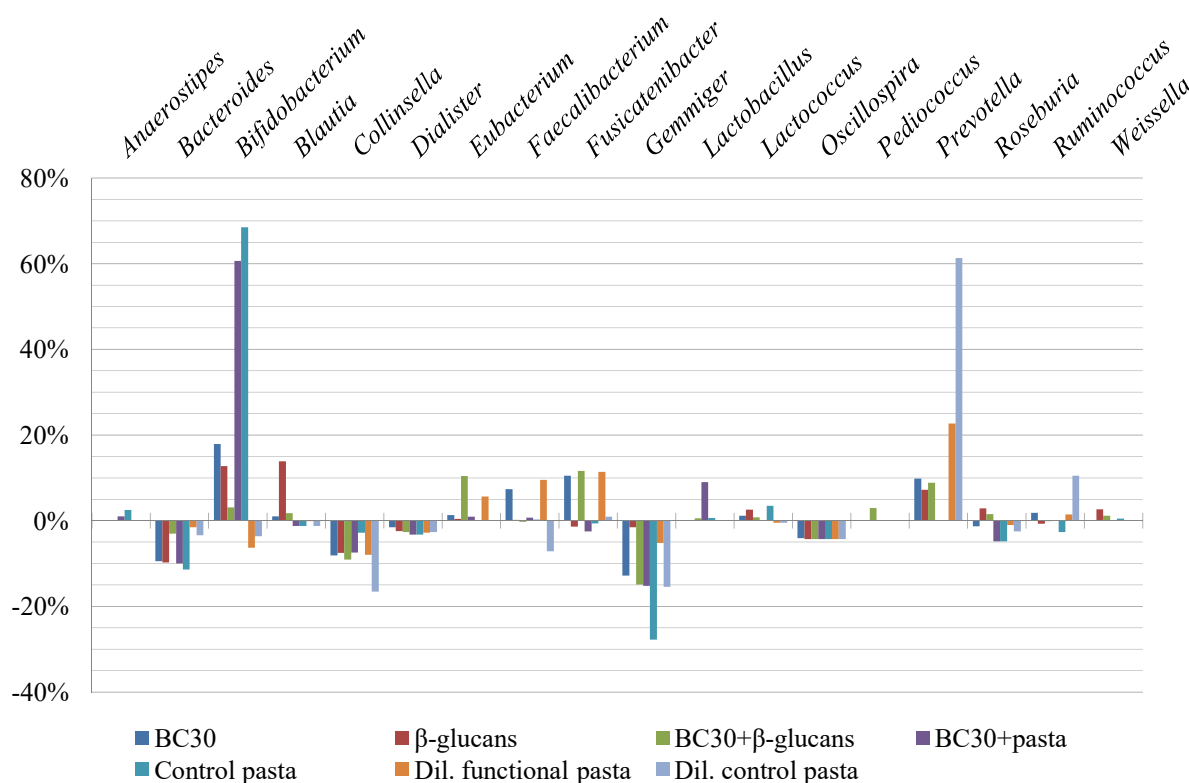


Figure 4.3. Modulations of microbial genera after 72 hour-fermentations with the different dietary treatments, on the microbiota from healthy children.

Regarding these most relevant modulations, a massive increase in *Bifidobacterium* was shown after administration of pasta with BC³⁰ added and control pasta, and a remarkable increase was also observed after treatment with BC³⁰, and β-glucans. The second most relevant increase was observed for *Prevotella*, that markedly responded to the interventions with the diluted control and functional pasta.

Blautia increased after administration of β-glucans, while *Eubacterium* increased after the combination of BC³⁰ and β-glucans, and the functional pasta. *Faecalibacterium* was positively modulated by BC³⁰ and the diluted functional pasta, and *Fusicatenibacter* was increased after treatment with BC³⁰, BC³⁰+β-glucans and the diluted functional pasta. Interestingly, *Lactobacillus* increased after administration of BC³⁰+pasta. Finally, an increase in *Ruminococcus* was observed after treatment with the diluted control pasta.

These experiments with TIM-2 model allowed to explore the effects of the administration of the functional pasta and to compare them with the effects due to its functional ingredients, in order to investigate the contribution of each ingredient in the final effect observed for the functional pasta. In the healthy microbiota the diluted functional pasta caused remarkable increases in *Eubacterium*, *Faecalibacterium*, *Fusicatenibacter*, and *Prevotella*. Considering *Fusicatenibacter*, which increased after BC³⁰, BC³⁰+ β -glucans and the diluted functional pasta treatments, but not after β -glucans or high concentrations of starch, it was possible to suppose that the increase in this genus could be attributed to the probiotic strain. In the same way, the increase in *Eubacterium* in the functional pasta, and in the combination of BC³⁰+ β -glucans appeared to be attributed mostly to the probiotic strain, as it was observed also with respect to the increase in *Faecalibacterium*. Thus, in the healthy microbiota the probiotic strain appeared to promote the growth of *Fusicatenibacter*, *Faecalibacterium* and *Eubacterium*. In particular, increases in *Faecalibacterium* and *Eubacterium* could represent beneficial effects of the functional pasta. Finally, the growth of *Prevotella* after administration of the functional pasta could be attributed to the diluted starch, and also to the two functional ingredients.

Regarding pre-diabetic microbiota, sixteen genera were observed to be modulated by the tested treatments (Figure 4.4). Some of the groups were modulated in the same way for each treatments, i.e. *Collinsella*, and *Gemmiger* were always decreased. The 14 genera that resulted increased after the treatments with the nutritional intervention were reported in Table 4.1b.

Most important modulations involved genera *Bacteroides*, *Bifidobacterium*, *Blautia*, *Eubacterium*, *Lactobacillus*, *Prevotella* and *Ruminococcus*.

Again, the most relevant modulation was that related to genus *Bifidobacterium*, markedly increased after treatment with the highest doses of starch (control pasta and pasta added with BC³⁰), followed by treatments with the diluted functional pasta and with BC³⁰+ β -glucans. Another modulation particularly pronounced was the increase of *Lactobacillus* after administration of BC³⁰+pasta, as already observed in a minor extent in the healthy microbiota. *Bacteroides* resulted mainly increased after treatment with β -glucans and the diluted functional pasta. *Blautia* levels increased primarily after administration of BC³⁰, β -glucans, and BC³⁰+ β -glucans, while *Eubacterium* increased after treatment with β -glucans, and BC³⁰+ β -glucans. *Prevotella* and *Ruminococcus* levels resulted increased after intervention with BC³⁰ and the diluted control pasta, and *Ruminococcus*, resulted increase also due to the functional pasta.

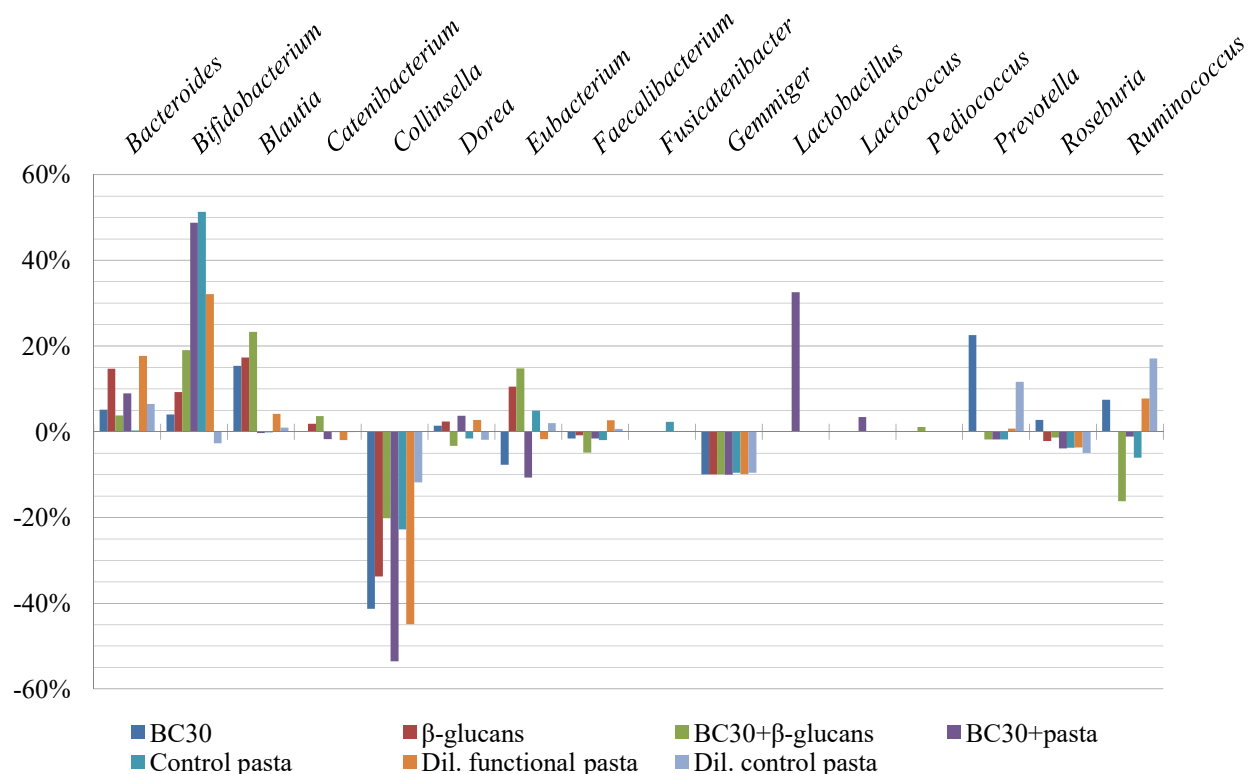


Figure 4.4. Modulations of microbial genera after 72 hour-fermentations with the different dietary treatments, on the microbiota from pre-diabetic children.

The intervention with the functional pasta led to increases in the genera *Bacteroides*, *Bifidobacterium*, and *Ruminococcus*. With the intent to relate effects of the functional pasta with effects of its ingredients, increase in *Bacteroides* appeared to be mainly attributed to β-glucans. The strong increase in *Bifidobacterium* could be caused by the starch, even if a minor bifidogenic potential was presented also by the two functional ingredients. Increase in *Ruminococcus* levels could be attributed both to the probiotic strain and the diluted starch.

Regarding diabetic microbiota, 19 genera appeared to be modulated after treatment with the different interventions (Figure 4.5), five of which decreased, namely *Coprococcus*, *Dialister*, *Faecalibacterium*, *Lachnoclostridium* and *Oscillospira*.

The 14 increased genera were reported in Table 4.1c. Also in the case of diabetic microbiota, the most modulated genus was *Bifidobacterium*, which markedly increased after administration of all tested interventions, and in particular of the control pasta and the pasta added with the probiotic strain. Also *Collinsella* was positively modulated by almost every treatment, with the highest increase observed after administration of BC³⁰+pasta.

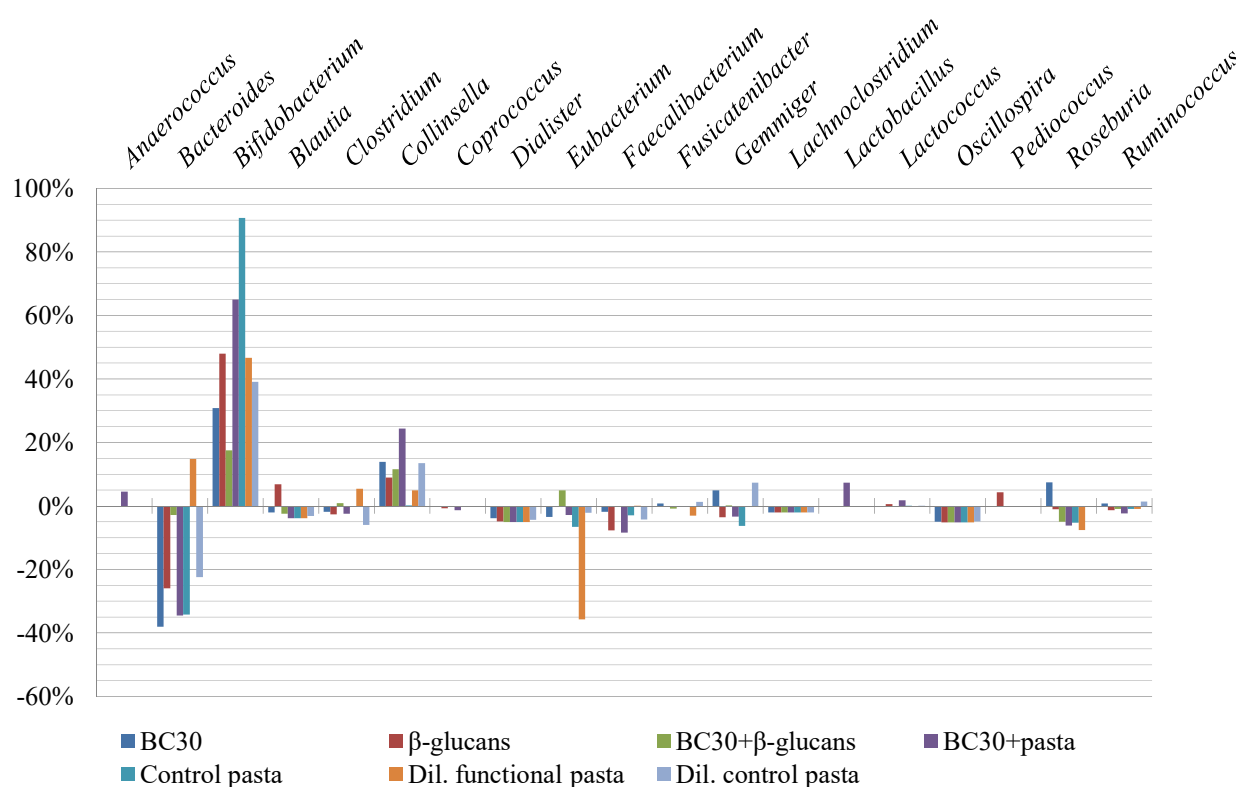


Figure 4.5. Modulations of microbial genera after 72 hour-fermentations with the different dietary treatments, on the microbiota from diabetic children.

Lactobacillus was increased also in this case after treatment with the pasta with probiotic strain added, together with *Anaerococcus*. *Bacteroides* and *Clostridium* were increased after treatment with the diluted functional pasta. Other minor modulations involved increases in *Blautia*, following β -glucans intervention, *Eubacterium*, after BC³⁰+ β -glucans administration, *Gemmiger*, with BC³⁰ and the diluted control pasta, *Roseburia*, with BC³⁰.

In diabetic microbiota, the treatment with the functional pasta led to increases in *Bacteroides*, *Bifidobacterium*, *Clostridium* and *Collinsella*. Increases in *Bacteroides* and *Clostridium* did not appear to be directly related to one of the pasta components. Referring to *Bifidobacterium*, each ingredient of the pasta, i.e. the probiotic strain, the β -glucans and the starch, had a strong bifidogenic effect. Also for *Collinsella*, from the same family Actinobacteria, each component appeared to contribute to the increase observed for the functional pasta.

Results of *in vitro* fermentations gave a broad range of information regarding modulation of specific microbial groups following the different nutritional intervention. In many cases a specific treatment led to different responses in the microbiota of the three groups, i.e. different

patterns of microbial genera were increased in a specific microbiota (healthy, pre-diabetic, and diabetic), but not in another. For example, *Fusicatenibacter* increased after administration of the probiotic strain in the healthy microbiota, but not in the pre-diabetic or in the diabetic ones. This can be due to differences in the composition of the three microbiota at the baseline of the experiments, which led to alternative cross-feeding.

With respect to the microorganism that appeared related the healthy microbiota (i.e. *Gemmiger formicilis* and *Faecalibacterium prausnitzii*) and to the pre-diabetic and diabetic ones (*Bacteroides vulgatus* and *Bifidobacterium adolescentis*) at the beginning of the *in vitro* experiments, a positive output of a treatment could be the increase in a biomarker characteristic of the healthy state or a decrease in a biomarker considered as associated to a pre-pathological or pathological conditions. *G. formicilis* resulted decreased following almost every treatment in the healthy, pre-diabetic and diabetic microbiota. The exceptions were represented by the probiotic strain BC³⁰ and the diluted control pasta, that led to an increase in this microbial species in the diabetic group. Regarding *F. prausnitzii*, in the healthy microbiota it resulted increased following intervention with the probiotic strain BC³⁰ and the functional pasta, while in the pre-diabetic microbiota, an increase in this species was observed after administration of the functional pasta. In diabetic microbiota *F. prausnitzii* did not increase following any of the treatment.

Regarding the species that at the beginning of the *in vitro* study resulted associated to the pre-diabetic and diabetic microbiota, treatments leading to a decrease in *B. vulgatus*, that could represent a positive effect, were the control pasta in the healthy and diabetic microbiota, and the probiotic strain in the diabetic microbiota; in pre-diabetic microbiota all treatments led to an increase in this species (data not shown). Finally, regarding *B. adolescentis*, most of the treatment caused an increase in this species. Among these, treatments with the highest doses of starch, and most of all the control pasta caused the major increases. The pasta enriched with the probiotic strain led to a different effect in the pre-diabetic and diabetic microbiota; this aspect will be further discussed later. Overall, these results indicated that some treatments (e.g. the probiotic strain, the functional pasta), more than others (e.g. β -glucans, BC³⁰+ β -glucans) could promote the growth of the markers of the healthy condition or the decrease of the markers related to the pre-pathological/ pathological status. However, in most cases results were microbiota-dependent.

Table 4.1. Modulations of microbial genera following the different *in vitro* interventions; a) microbiota from healthy children, b) microbiota from pre-diabetic children, c) microbiota from diabetic children.

a.

HEALTHY	<i>Anaerostipes</i>	<i>Bifidobacterium</i>	<i>Blautia</i>	<i>Eubacterium</i>	<i>Faecalibacterium</i>	<i>Fusicatenibacter</i>	<i>Lactobacillus</i>	<i>Lactococcus</i>	<i>Pediococcus</i>	<i>Prevotella</i>	<i>Roseburia</i>	<i>Ruminococcus</i>	<i>Weissella</i>
BC ³⁰		↑ 17.9%	(↑) 1.0%	(↑) 1.3%	↑ 7.4%	↑ 10.5%		(↑) 1.2%		↑ 9.9%		(↑) 1.9%	
β-glucans		↑ 12.7%	↑ 13.9%					(↑) 2.6%		↑ 7.2%	(↑) 2.9%		(↑) 2.7%
BC ³⁰ + β-glucans		(↑) 3.1%	(↑) 1.8%	↑ 10.4%		↑ 11.6%			(↑) 2.9%	↑ 8.8%	(↑) 1.6%		(↑) 1.2%
BC ³⁰ +pasta	(↑) 1.0%	↑↑↑ 60.6%		(↑) 1.0%			↑ 9.0%						
Control pasta	(↑) 2.5%	↑↑↑ 68.5%						(↑) 3.5%					
Dil. functional pasta				↑ 5.6%	↑ 9.5%	↑ 11.4%				↑↑ 22.7%		(↑) 1.5%	
Dil. control pasta						(↑) 1.0%				↑↑↑ 61.3%		↑ 10.5%	

b.

PRE-DIABETIC	<i>Bacteroides</i>	<i>Bifidobacterium</i>	<i>Blautia</i>	<i>Catenibacterium</i>	<i>Dorea</i>	<i>Eubacterium</i>	<i>Faecalibacterium</i>	<i>Fusicatenibacter</i>	<i>Lactobacillus</i>	<i>Lactococcus</i>	<i>Pediococcus</i>	<i>Prevotella</i>	<i>Roseburia</i>	<i>Ruminococcus</i>
BC ³⁰	↑ 5.1%	(↑) 4.0%	↑ 15.4%		(↑) 1.4%							↑↑ 22.5%	(↑) 2.7%	↑ 7.5%
β-glucans	↑ 14.7%	↑ 9.2%	↑ 17.3%	(↑) 1.9%	(↑) 2.4%	↑ 10.5%								
BC ³⁰ + β-glucans	(↑) 3.8%	↑ 19.1%	↑↑ 23.3%	(↑) 3.7%		↑ 14.8%					(↑) 1.1%			
BC ³⁰ +pasta	↑ 9.0%	↑↑ 48.8%			(↑) 3.8%				↑↑ 32.6%	(↑) 3.4%				
Control pasta		↑↑ 51.3%				↑ 5.0%		(↑) 2.3%						
Dil. functional pasta	↑ 17.7%	↑↑ 32.2%	(↑) 4.2%		(↑) 2.8%		(↑) 2.7%							↑ 7.8%
Dil. control pasta	↑ 6.5%		(↑) 1.0%			(↑) 2.0%						↑ 11.7%		↑ 17.1%

c.

DIABETIC	<i>Anaerococcus</i>	<i>Bacteroides</i>	<i>Bifidobacterium</i>	<i>Blautia</i>	<i>Clostridium</i>	<i>Collinsella</i>	<i>Eubacterium</i>	<i>Fusicatenibacter</i>	<i>Gemmiger</i>	<i>Lactobacillus</i>	<i>Lactococcus</i>	<i>Pediococcus</i>	<i>Roseburia</i>	<i>Ruminococcus</i>
BC ³⁰			↑↑ 30.8%			↑ 13.9%			↑ 4.9%				↑ 7.4%	
β-glucans			↑↑ 48.0%	↑ 6.8%		↑ 8.9%						(↑) 4.3%		
BC ³⁰ + β-glucans			↑ 17.6%			↑ 11.6%	↑ 4.9%							
BC ³⁰ +pasta	(↑) 4.5%		↑↑↑ 65.0%			↑↑ 24.3%				↑ 7.4%	(↑) 1.8%			
Control pasta			↑↑↑ 90.7%											
Dil. functional pasta		↑ 14.8%	↑↑ 46.6%		↑ 5.5%	↑ 4.9%								
Dil. control pasta			↑↑ 39.1%			↑ 13.5%		(↑) 1.3%	↑ 7.3%					(↑) 1.4%

Modulations for a specific microbial group are indicated in the table if the difference in abundance (Time 72h-Time 0h), normalised to the control meal, is close to or > 1%.

(↑) increase corresponding to a value <5%.

↑ increase corresponding to a value between 5% and 20%.

↑↑ increase corresponding to a value between 20% and 60%.

↑↑↑ increase corresponding to a value > 60%.

Shared effects of the ingredients and pasta on certain bacterial groups in the different microbiota

As reported previously, many of the tested diets led to modulations that were microbiota-dependent. On the contrary, interestingly, some of the nutritional interventions led to modulations of specific microbial groups, that were shared in the healthy, pre-diabetic and diabetic microbiota or in two of them. In particular, modulations shared in the three microbiota resulted the most interesting, because particularly informative of the effects of a certain dietary intervention.

Bacillus coagulans BC³⁰ appeared to have a bifidogenic effect, that was much more pronounced on healthy (17.9%) and diabetic microbiota (30.8%) than the pre-diabetic one (4.0%) (Figure 4.6a). In healthy and pre-diabetic microbiota, BC³⁰ markedly increased *Prevotella* levels (9.9% and 22.5%, respectively), and to a minor extent, *Ruminococcus* levels (1.9% and 7.5%, respectively). Finally, increases of *Roseburia* were observable after administration of the probiotic strain to the pre-diabetic and diabetic groups (2.7% and 7.4%). β -glucans, also, had a marked effect on *Bifidobacterium*, on the healthy, pre-diabetic and diabetic microbiota (12.7%, 9.2% and 48.0%) (Figure 4.6b). Moreover, a shared effect on the three microbiota was observed regarding increases of *Blautia* (13.9%, 17.3%, 6.8%). When *Bacillus coagulans* BC³⁰ and β -glucans were administered together, increases in *Bifidobacterium* were observed for the three microbiota (3.1%, 19.1% and 17.6%) , together with increases in *Eubacterium* (10.4%, 14.8% and 4.9%) (Figure 4.6c). Increases of *Blautia* and *Pediococcus* were observed in healthy and pre-diabetic group (1.8% and 23.3% for *Blautia*, 2.9% and 1.1% for *Pediococcus*).

Thus, after the intervention with the two functional ingredients, BC³⁰ and β -glucans, administered separately or in combination, an increase in *Bifidobacterium* was observed in the three microbiota. If a bifidogenic effect was already known for β -glucans, *in vitro* (Hughes *et al.*, 2008; Kedia *et al.*, 2009) and *in humana* (Mårtensson *et al.*, 2005; Mitsou *et al.*, 2010), this effect was not reported for the probiotic strain BC³⁰ in previous studies (Honda *et al.*, 2011; Nyangale *et al.* 2015). Regarding the increase in *Blautia* observed after administration of β -glucans, a previous *in humana* study showed an increase in this genus following ingestion of a whole grain barley flakes containing a high amount of β -glucans (14.1%) (Martínez *et al.*, 2013a). Another interesting trend observed in the three microbiota was the increase in *Eubacterium* spp., and in particular in *Eubacterium rectale*, after the administration of BC³⁰+ β -glucans. In the healthy microbiota, increases in *Eubacterium* appeared to be due mainly to the probiotic strain, while in the pre-diabetic microbiota the effect could be attributed to the β -

glucans; in the diabetic microbiota no direct connection with one of the two functional ingredients was observed. An increase in *E. rectale* after combined administration of the probiotic strain BC³⁰ and a probiotic (i.e. FOS or GOS) has been previously reported in literature (Nyangale *et al.*, 2014).

The pasta with added BC³⁰ led to a marked increase of *Bifidobacterium* (60.6%, 48.8% and 65.0%), and interestingly also *Lactobacillus* was increased after this nutritional treatment in the three microbiota (9.0%, 32.6% and 7.4%). *Lactococcus* resulted increased in pre-diabetic and diabetic microbiota (3.4% and 1.8%) (Figure 4.7a). Also the control pasta, as previously reported had a strong bifidogenic effect in the three microbiota (68.5%, 51.3% and 90.7%). (Figure 4.7b). Thus, the most relevant modulation after administration of the pasta added with the probiotic strain and the control pasta (i.e. treatment with the highest doses of starch) was the marked growth in *Bifidobacterium*. Increases in *Bifidobacterium* population after administration of starch was previously reported in literature (Liu *et al.*, 2015). Moreover, the enormous increase in *Bifidobacterium* observed with these diets could have been amplified from the fact that investigated pasta were whole grain products. Examples of bifidogenic effect of whole grain cereal products from wheat (Costabile *et al.*, 2008), or other cereal, e.g. maize (Carvalho-Wells *et al.*, 2010) have been reported in literature; in these studies the bifidogenic effects obtained from WG products were more pronounced to that observed with the control, i.e. non-whole grain cereals. At the species level, evidences from literature reported that a certain species, *B. adolescentis*, is reported to increase following administration of starch, in particular R2 and R4 type starch (Martínez *et al.*, 2010; Venkataraman *et al.*, 2016). In the present study, increases in *B. adolescentis* were observed after administration of the pasta enriched with the probiotic strain in the healthy microbiota, and of the control pasta in the healthy, pre-diabetic and diabetic microbiota. However, this species was not the only species involved in the massive increase observed for *Bifidobacterium* genus, as further explained in the following paragraph.

At the end of the *in vitro* interventions *Bifidobacterium* represented the dominant population (>50% of the total population) (data not shown). It has to be considered that such a high increase in this microbial genus may be less pronounced *in vivo*, due to the fact that the children would have a varied diet; thus, the bifidogenic effect of these meals could be exaggerated in TIM-2.

Another very interesting aspect was the increase in *Lactobacillus* spp. in the three microbiota, following administration of the pasta added with the probiotic strain. Since this effect was not observed after treatment with the control pasta, it appeared to be related to the presence of the probiotic strain; in any case, *Lactobacillus* was not increased after treatment with BC³⁰ alone, so

probably, the stimulation of this genus was due to the synergistic effect of the starch and the probiotic strain. This aspect will be further discussed in the following paragraph.

Different modulations were obtained for the functional pasta. An increase in *Bacteroides* was observed for the pre-diabetic and diabetic microbiota (17.7% and 14.8%) as well as an increase in *Bifidobacterium* was observed for the same microbiota (32.2% and 46.6%). *Faecalibacterium* was increased in healthy and pre-diabetic groups (9.5% and 2.7%) and the same was for *Ruminococcus* (1.5% and 7.8%) (Figure 4.7c).

The diluted control pasta, which presented a lower dose of starch with respect to the control pasta, had an effect on healthy and pre-diabetic microbiota on *Prevotella* (61.3% and 11.7%) and *Ruminococcus* (10.5% and 17.1%); *Ruminococcus* levels were also slightly increased in the diabetic microbiota (1.4%) (Figure 4.7d). In particular, the species *Ruminococcus bromii* was responsible of the increase observed for *Ruminococcus* genus after administration of starch. This result was consistent with previous findings that indicated *R. bromii* as a key species in the degradation of resistant starch type 3 in the human colon (Walker *et al.*, 2011; Ze *et al.*, 2012). Increases in this microbial genus or species were not observed after the administration of higher doses of starch, i.e pasta enriched with the probiotic strain and control pasta, probably because of the massive increase in *Bifidobacterium* spp.

In conclusion, in the analysed microbiota, interventions with tested treatments resulted in modulations of microbial *taxa* that are consistent with previous literature about specific diet-microbial group relationship. Furthermore, in general, certain tested treatments led to positive modulations of bacterial groups that are considered as beneficial at the gut microbiota level, in term of healthy promoting organisms (i.e. *Bifidobacterium* and *Lactobacillus* spp.), and producers of beneficial metabolites, as butyrate (i.e. *Eubacterium rectale*, *Roseburia* and *F. prausnitzii*).



Figure 4.6. Modulations of microbial genera after 72 hour-fermentations with a) the probiotic strain BC³⁰ b) the β -glucans and c) the probiotic strain BC³⁰ + β -glucans, on the microbiota from healthy (in green), pre-diabetic (in yellow) and diabetic (in red) children.

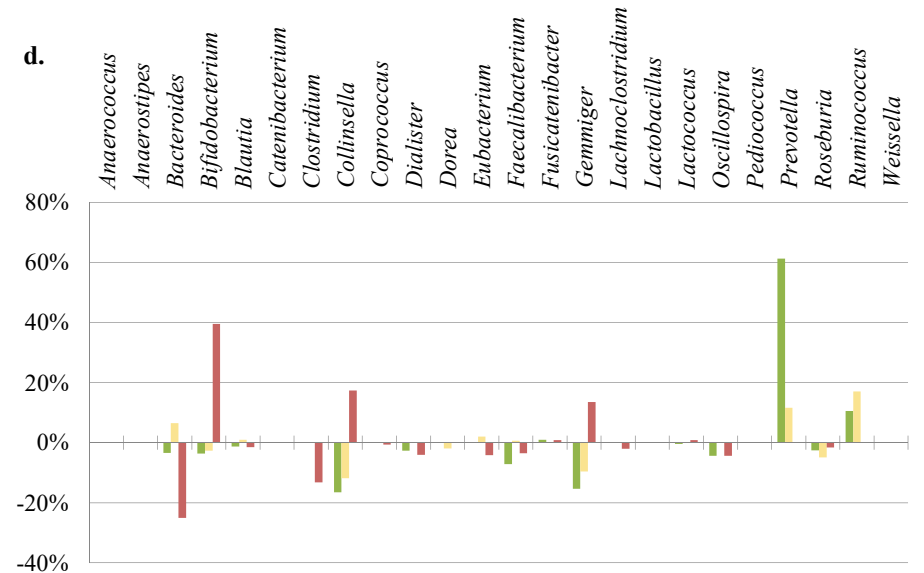
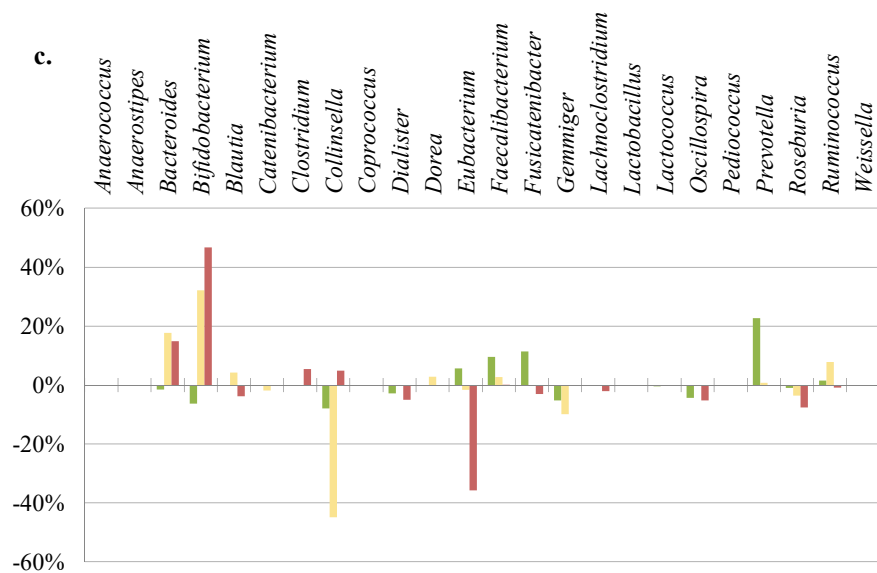
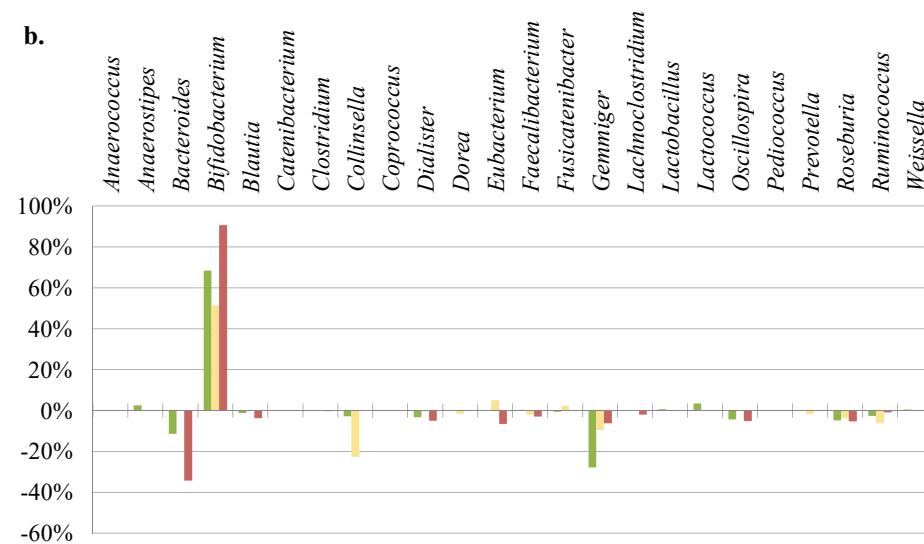
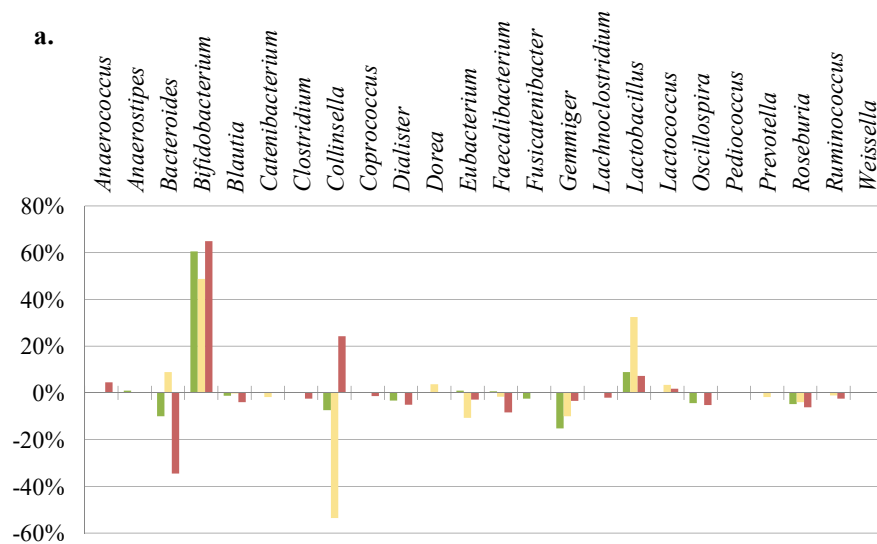


Figure 4.7. Modulations of microbial genera after 72 hour-fermentations with a) the pasta enriched with the probiotic strain BC³⁰, b) the control pasta, c) the functional pasta and d) the diluted control pasta, on the microbiota from healthy (in green), pre-diabetic (in yellow) and diabetic (in red) children.

Analyses at the species level for Bifidobacterium genus following treatment with the highest concentration of starch

Given the differences in *Bifidobacterium* abundances at time 0 hours in the healthy, pre-diabetic and diabetic microbiota, and in particular considering that *B. adolescentis* was observed to be characteristic of the pre-diabetic and diabetic group, it was interesting to investigate *Bifidobacterium* at the species level following treatments that caused the most marked increase in this microbial genus (control pasta and pasta added with BC³⁰) in order to understand which species were stimulated from the treatments.

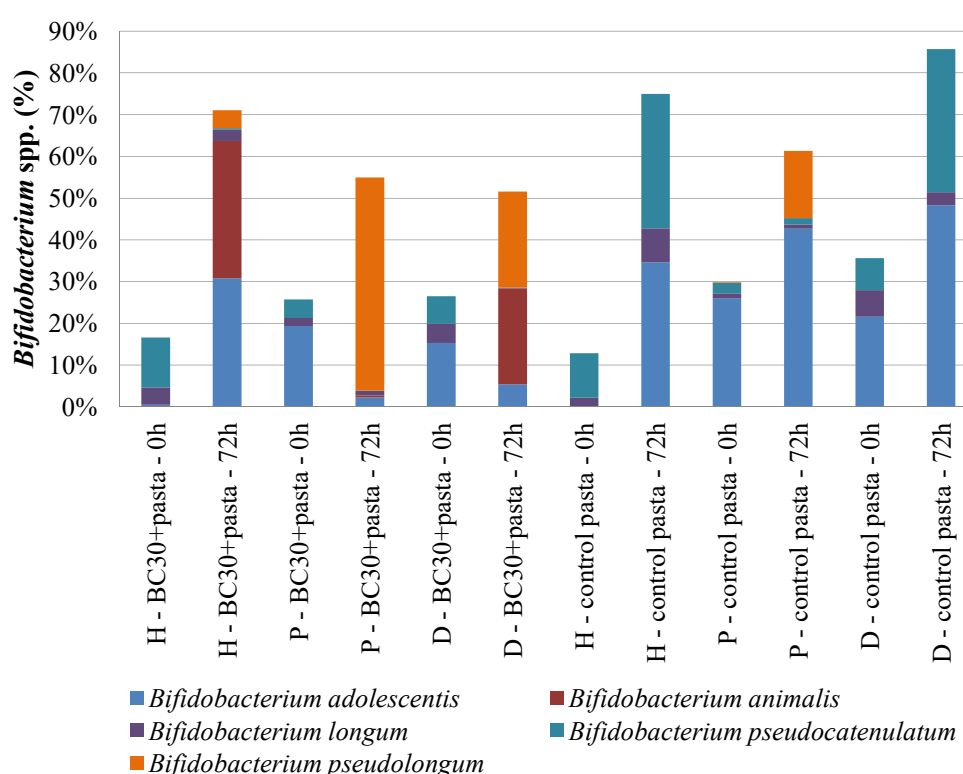


Figure 4.8. *Bifidobacterium* species at the beginning (time 0 hours) and at the end (time 72 hours) of the intervention with the probiotic pasta and the control pasta, on the microbiota from healthy (H), pre-diabetic (P) and diabetic (D) children.

Analyses at the beginning (time 0 hours) and at the end (time 72 hours) of the nutritional treatments with these two pastas showed that four species were mainly involved in the massive increase of this microbial group, namely *B. adolescentis*, *B. animalis*, *B. pseudocatenulatum*, *B. pseudolongum* (Figure 4.8).

Among these four species, most interesting results were related to *B. adolescentis* and *B. animalis*. Regarding *B. adolescentis*, considering the hypothesis of this species as a putative marker of a pre-pathological/pathological condition, its increase would not be beneficial.

Administration of the control pasta led to an increase in this species in each of the three microbiota, and an increase was observed also after treatment with the pasta added with BC³⁰, but just in the healthy group. On the contrary, interestingly, in the pre-diabetic and diabetic groups *B. adolescentis* resulted decreased after the intervention with the probiotic pasta. Thus, a treatment with the probiotic pasta, that could reduce this microbial species would be potentially advisable.

B. animalis was increased after intervention with pasta with BC³⁰ added, especially in control and diabetic microbiota, while it was not present in the three microbiota after treatment with the control pasta. Consequently, its growth would appear related to BC³⁰ administration; however this species did not increase after administration of the other meals containing BC³⁰ (the probiotic strain itself, the combination of BC³⁰+ β -glucans, the diluted functional pasta). Thus, probably, the increase in *B. animalis* was due to a synergistic effect of BC³⁰ and the pasta (high concentration of starch). Growth of *B. animalis* has been reported after administration of a probiotic mixture (*Lactobacillus* and *Bifidobacterium* strain) together with a prebiotic, i.e. inulin, in rats presenting colitis (Schultz *et al.*, 2004). Moreover, it has been reported that *B. animalis* could promote growth of *Lactobacillus* species in *in vitro* fermentations, through production of exopolysaccharides (Salazar *et al.*, 2009). This could explain the increase in *Lactobacillus* observed after administration of the pasta added with the probiotic strain (Figure 4.7a).

Finally, *B. pseudolongum* increased after intervention with the probiotic pasta, in a marked trend for the pre-diabetic and diabetic group, and after treatment with the control pasta in the pre-diabetic group. *B. pseudocatenulatum* markedly increased after administration of the control pasta in the healthy and diabetic microbiota, while it remained at a low level in the pre-diabetic group; treatment with the probiotic pasta decreased this species in each of the three microbiota.

SCFA production

Production of acetate, propionate and butyrate was measured by gas chromatography on samples collected during *in vitro* fermentations, and results on samples collected at the end of fermentation (time 72 hours) are presented.

Interventions with the different ingredients caused different relative proportions among SCFA (% of total SCFA) and amounts, and this was observed for any of the three microbiota, healthy (Figure 4.9a, b), pre-diabetic (4.10a, b) and diabetic. (fig 4.11a, b).

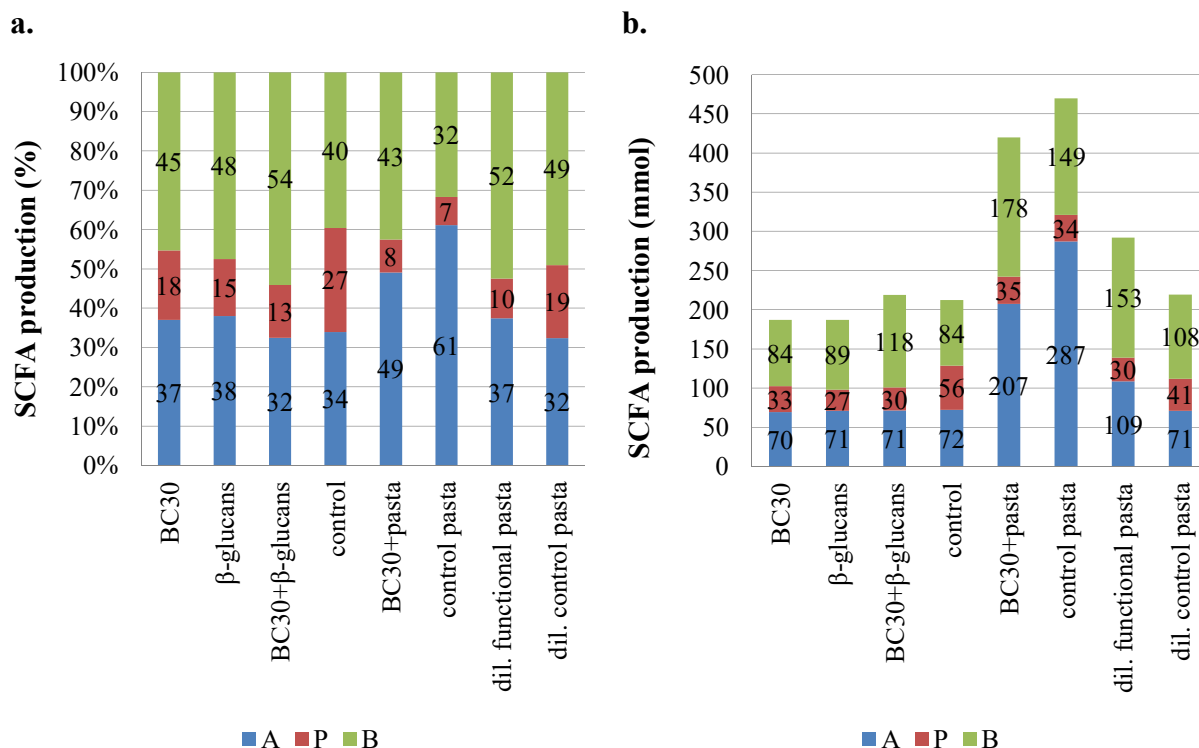


Figure 4.9. Acetate, propionate and butyrate production in the microbiota from healthy children after 72 hours of fermentation with the different dietary treatments, expressed as percentages of total SCFA production (a), and absolute amounts (b).

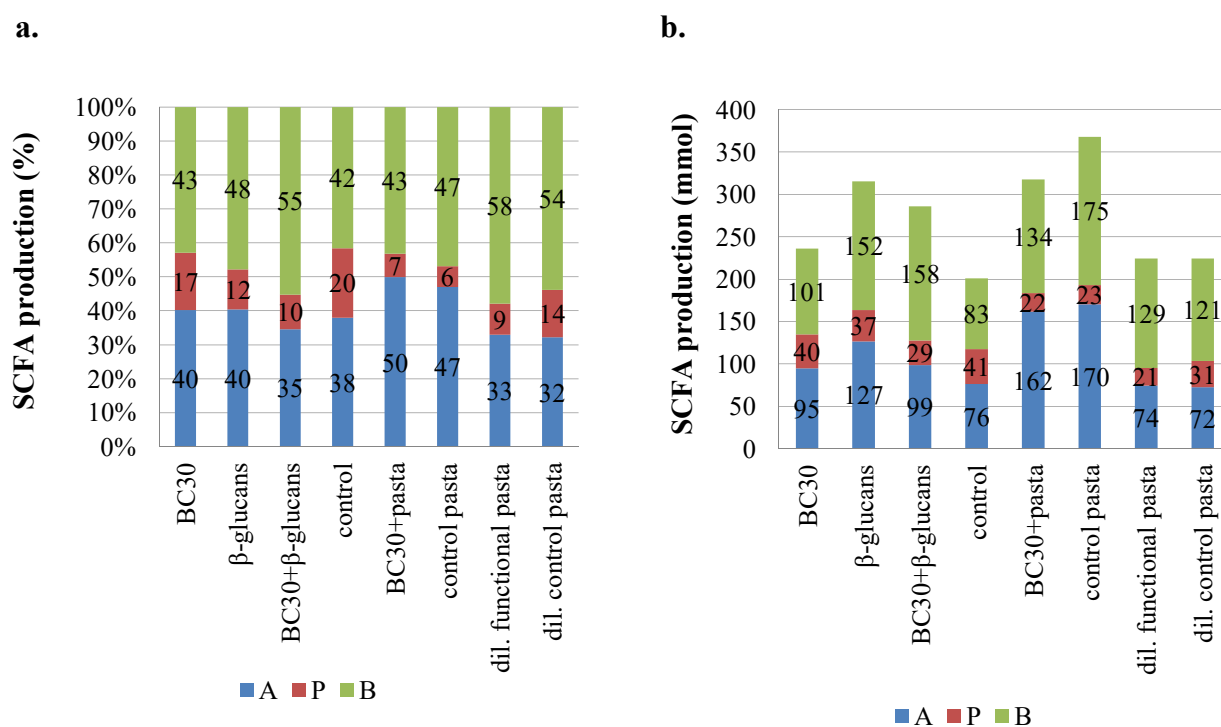


Figure 4.10. Acetate, propionate and butyrate production in the microbiota from pre-diabetic children after 72 hours of fermentation with the different dietary treatments, expressed as percentages of total SCFA production (a), and absolute amounts (b).

In the healthy group (Figure 4.9), butyrate was markedly the dominant produced metabolite (about 50% or more of total SCFA production) following administration of the combination of BC³⁰ and β -glucans, and of the diluted functional and control pasta. On the contrary, acetate was the main product obtained following fermentation with the pasta added with the probiotic strain and, most of all, with the control pasta.

Also in the pre-diabetic group (Figure 4.10), butyrate was the metabolite produced in highest proportions following administration the combination of BC³⁰ and β -glucans, and of the diluted functional and control pasta. Moreover, also β -glucans led to a similar production. In the pre-diabetic microbiota, acetate was the main product of fermentation with the pasta added with the probiotic strain, while the control pasta led to a comparable production of acetate and butyrate.

Finally, in the diabetic group (Figure 4.11), butyrate was the metabolite produced in the highest ratios after administration of β -glucans, the combination of BC³⁰ and β -glucans, and of the diluted functional and control pasta. Acetate was the main product of fermentations with the pasta added with the probiotic strain and the control pasta.

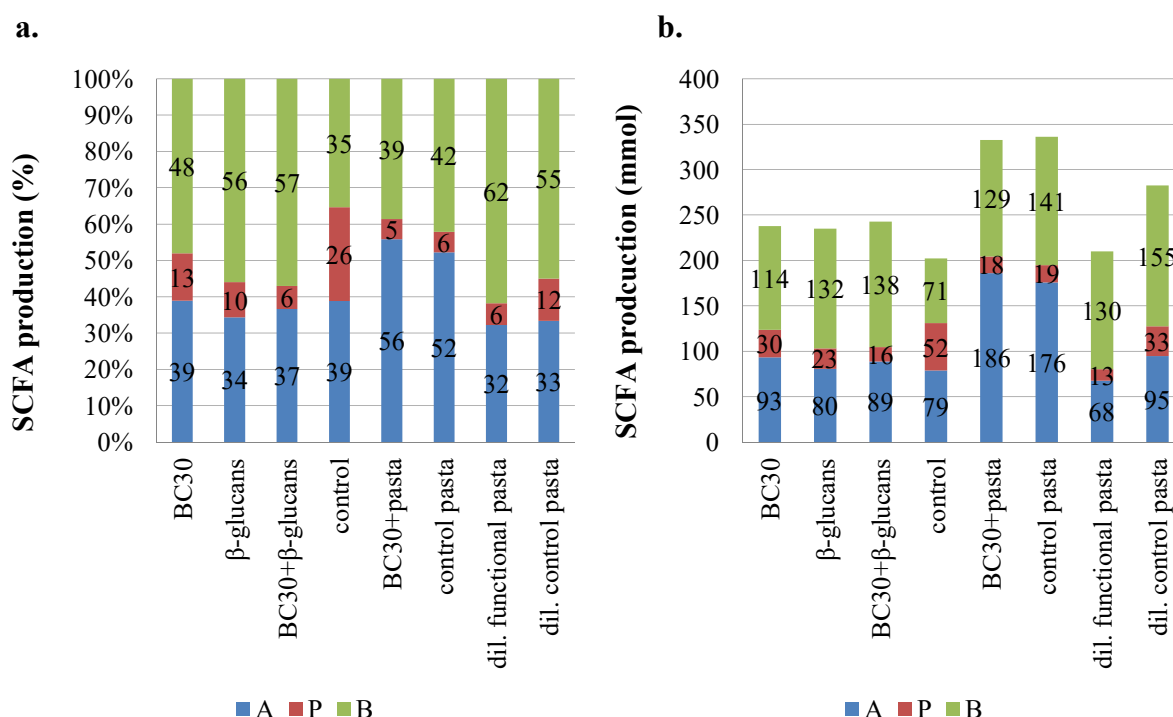


Figure 4.11. Acetate, propionate and butyrate production in the microbiota from diabetic children after 72 hours of fermentation with the different dietary treatments, expressed as percentages of total SCFA production (a), and absolute amounts (b).

Obtained results showed that, in general, the relative proportions of SCFA observed after treatment with a specific diet appeared comparable in the three microbiota (Figure. 4.12). The ratio observed for the control meal at the end of the fermentations was considered as a reference and was 37:24:39, with acetate and butyrate present in equal proportions. Acetate and butyrate were the main products of all fermentations, while propionate was in the lowest relative ratio, after any dietary treatment, in the three microbiota. In particular, butyrate was the principal metabolite at the end of the treatment with most of diets, with highest proportions obtained after treatment with the combination of BC³⁰ and β -glucans, the diluted functional and control pastas, in the three microbiota. Treatment with β -glucans also caused a high butyrate production in pre-diabetic and diabetic microbiota. On the contrary, acetate was the major metabolite revealed after treatment with the pasta enriched in the probiotic strain and the control pasta, i.e. the meals at the highest concentration of starch. An exception was represented by the control pasta treatment, which led to an equal production of acetate and butyrate in the pre-diabetic microbiota.

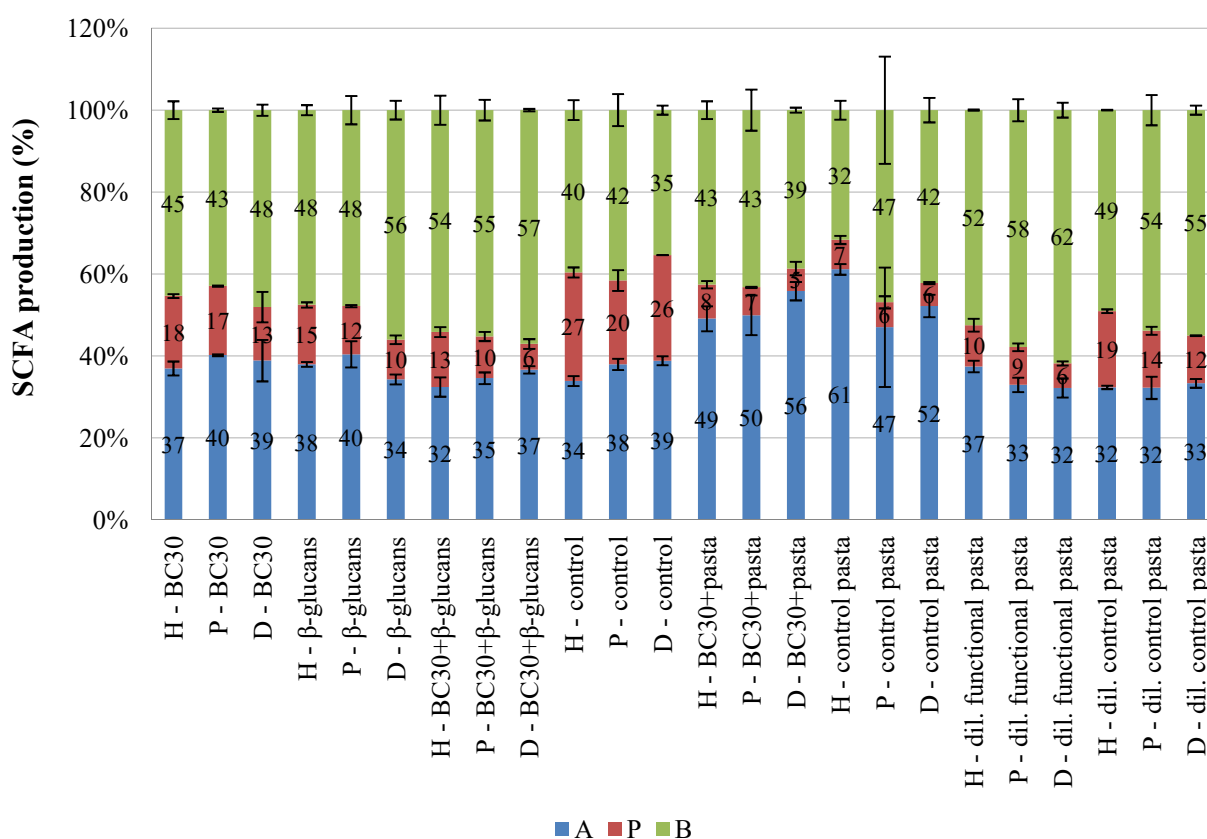


Figure 4.12. Ratios among acetate, propionate and butyrate (expressed as percentages of total SCFA production), in the microbiota from healthy, pre-diabetic and diabetic children, after 72 hours of fermentation with the different dietary treatments.

Considering absolute amounts, the highest production of total SCFA was observed after the interventions that included the highest dose of starch (control pasta and pasta added with BC³⁰), in control, pre-diabetic and diabetic microbiota (Figure 4.13a). Moreover, in the pre-diabetic microbiota, also and β -glucans led to similarly high average values (more than 300 mmol). The fact that a high dose of starch caused the highest amount of SCFA produced is consistent with literature, since starch is considered to provide the largest proportion of energy at the colon level (Duncan *et al.*, 2003).

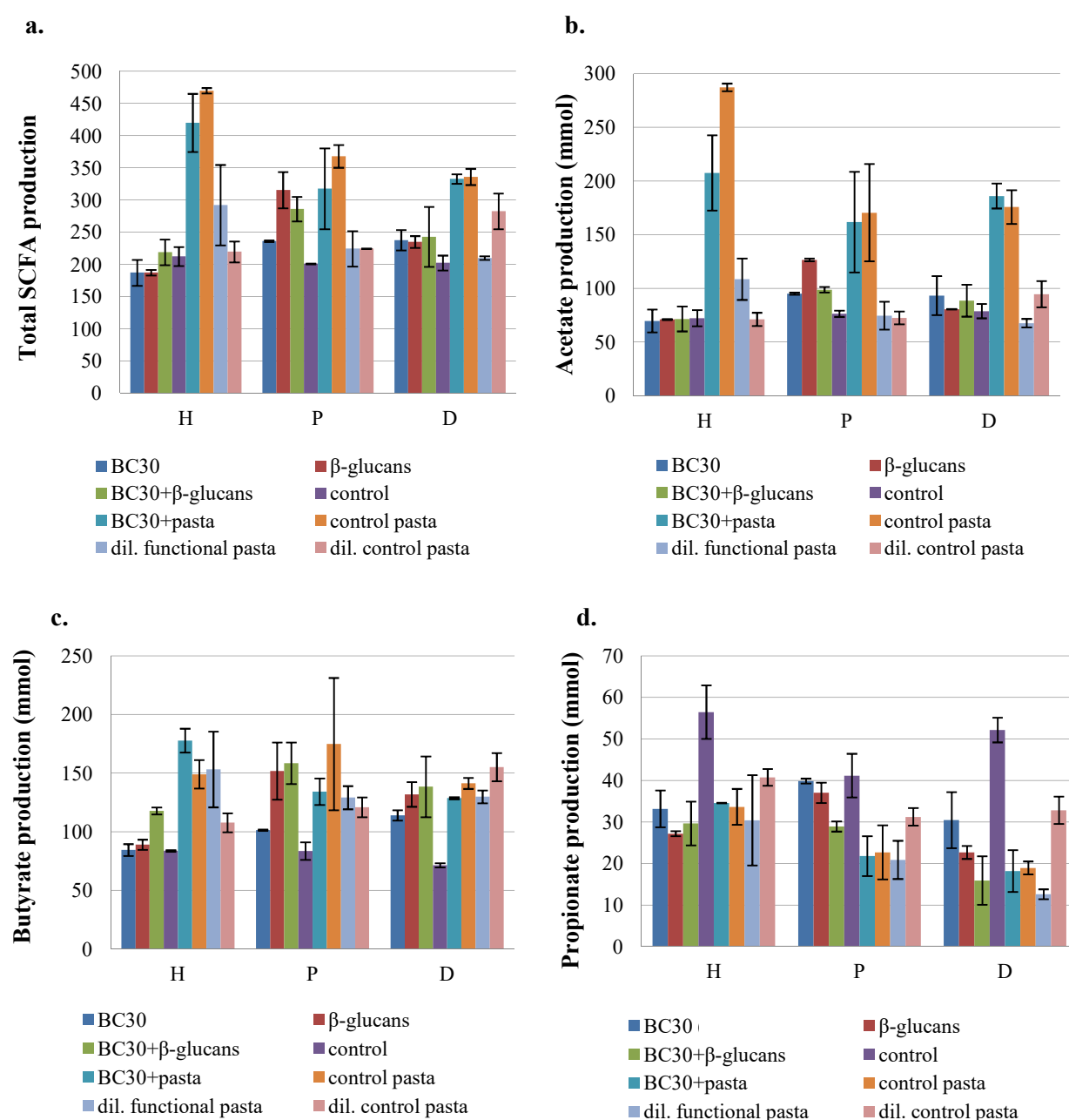


Figure 4.13. SCFA production after 72 hours of fermentations with the different dietary treatments, in the microbiota from healthy (H), pre-diabetic (P) and diabetic (D) children. In particular: a) total SCFA, b) acetate, c) butyrate and d) propionate production.

Beside leading to the highest production of total SCFA, administration of the two pasta led to the highest production of acetate, in term of average amounts (> 150 mmol), in each of the three microbiota (Figure 4.13b). Maximum production was observed in the healthy microbiota after treatment with the control pasta (287 mmol).

Referred to butyrate, common trends for a specific meal on the three different microbiota were not found (Figure 4.13c). Considering absolute amounts, in the healthy group butyrate production was at the highest levels after treatment with the pasta added with BC³⁰, and high values were obtained also with the control pasta and the diluted functional pasta. It was also interesting to note that, in the healthy group, the probiotic strain or the β -glucans, when administered separately, led to a production comparable to the one obtained with the control meal (84-89 mmol), while, when contemporarily present, they remarkably increased butyrate production (118 mmol).

In the pre-diabetic group, the β -glucans, the combination of the probiotic strain and β -glucans, and the control pasta, led to the highest average butyrate production. In this case, a high butyrate level following combined administration of the probiotic strain and the β -glucans was comparable to what obtained after treatment of the β -glucans alone. Thus, it appeared that in the pre-diabetic microbiota the prebiotic component mainly contributed to the production of butyrate, when the two functional ingredients were tested together.

Finally, for the diabetic group, almost every treatment (excluded the control pasta) led to a high butyrate production, with the maximum average value obtained for the diluted control pasta.

Regarding propionate production, the highest absolute amounts were produced after treatment with the control meal, in healthy (56 mmol) and diabetic (52 mmol) microbiota. For pre-diabetic microbiota, slightly lowest level of propionate were produced (41 mmol), and they were similar to those obtained following administration with the probiotic strain and β -glucans (Figure 4.13d).

In the contest of T1D, conditions leading to the highest production of butyrate would represent, in principle, the best output at which aiming, since butyrate is considered a beneficial metabolite that contributes to colon health, through the improvement of gut integrity (Brown *et al.*, 2011). In particular, a high butyrate production could be considered as both absolute amounts or proportions in the ratio with acetate and propionate. Results showed that not in all cases a high butyrate absolute amount matched with a condition in which butyrate was at the highest proportion (Figure 4.12 and Figure 4.13c). Thus, the highest yield in butyrate production can be considered the one that is based on both parameter. In this perspective, the functional pasta

provided the highest yield in butyrate in healthy and diabetic conditions, while BC³⁰+ β -glucans gave the highest yield in pre-diabetic microbiota.

On the contrary, treatments leading to a high production of acetate, that overcome butyrate and propionate production, as the diets at a high concentration of starch in the present study (i.e. the probiotic pasta and the control pasta) could be considered as not so beneficial. In particular, in diabetes contest, it was recently proposed that acetate could act as a promoter for the metabolic syndrome (Perry *et al.*, 2016); thus, diets leading to a high production of acetate could not be proper for pre-diabetic and diabetic children. As a comment regarding dietary fibres, even considering their beneficial properties, there is a lack of studies investigating their effects in children and adolescents (Edwards *et al.*, 2015), and a study related to diabetes in an animal model reported that a cereal diet could act as a promoter for T1D (Patrick *et al.*, 2013).

Of course, these comments about positive or negative effects of the tested treatments from a clinical point of view are speculative, and still far to be considered for the planning of an *in humana* trial in T1D. For example, the small size of the pools in the present study, i.e. couples of children, represented a limit of the work. For this reason, further investigations on microbiota from large number of donors will be necessary.

Merging microbial composition and SCFA following dietary interventions

As previously reported, a marked increase in *Bifidobacterium* was observed following the administration of the pasta enriched with the probiotic strain and the control pasta. Moreover, after the same two treatments, that included the highest concentration of starch, acetate production was at the highest levels. This was observed considering both acetate proportions (Figure 4.12) and absolute amounts (Figure 4.13b). Since *Bifidobacterium* spp. mainly produce acetate and lactate with their carbohydrate degradation metabolism (De Vuyst *et al.*, 2014), the two findings appeared to be connected one to each other. Only in one case, i.e. treatment with the control pasta in the pre-diabetic group, acetate was not the principal metabolite; in fact, acetate and butyrate amounts were at a similar level in this condition (Figure 4.12). Interestingly, observing microbiota composition in this specific condition, a high abundance in *Eubacterium*, and specifically in *E. rectale* (15.6%) one of the most abundant butyrate producers in human gut (Louis *et al.*, 2010) was detected, and this could explain the high butyrate level observed.

Considering butyrate production, different treatments in the three microbiota led to highest amounts (Figure 4.13c). In healthy microbiota, the pasta added with the probiotic strain, the control pasta and the diluted functional pasta were related to highest production in term of

amounts, as could be expected from the presence of starch and the previously reported high butyrate production from starch (Kovatcheva-Datchary *et al.*, 2009; Venkataraman *et al.*, 2016). High levels of butyrate observed after treatment with the control pasta could be due to increases in *Anaerostipes* (2.53%); and in particular in *Anaerostipes hadrus*, a butyrate producing species (Allen-Vercoe *et al.*, 2012). It has been reported that strains of *A. hadrus* can utilise acetate to produce butyrate (Allen-Vercoe *et al.*, 2012). Moreover, cross-feeding between species of the genus *Bifidobacterium* and acetate-converting butyrate producers have been already reported after probiotic administration (Falony *et al.*, 2006). Thus, a cross-feeding mechanism could be responsible of the observed high butyrate production also in the present study and explained the bifidogenic/butyrogenic effect observed. Basing on the same logic, butyrate production after intervention with the pasta enriched with the probiotic strain could be related to an increase in *Anaerostipes* and *Eubacterium* (both increased of approximately 1%), while increases observed following administration of the diluted functional pasta could be supported by the increase in *Eubacterium* (5.6%) and *Roseburia* (3.8%). This last treatment also caused the highest proportion of butyrate (Figure 4.12) in the ratio with acetate and propionate, thus representing the treatment leading the best butyrate yield. Regarding the other two treatments leading to the highest proportion of butyrate, high proportion following BC³⁰+ β -glucans administration could be related to an increased in *Eubacterium* (10.4%) and *Roseburia* (6.4%), while *Roseburia* could be potentially responsible of the butyrogenic effect following the diluted control pasta administration (2.3%). Considering butyrate production following BC³⁰+ β -glucans administration, in the healthy microbiota, it was reported in the previous paragraph that the probiotic strain or the β -glucans, when administered separately, did not increase butyrate production more than the control meal (84-89 mmol), while when contemporarily present, they remarkably increased butyrate production (118 mmol). This observed synergistic effect could be connected, from a compositional point of view, to the genus *Eubacterium*; in fact, this microbial group, that includes some of the most important butyrate producers, was not increased after the separated treatments with the two functional ingredients, but it markedly increased after their combined administration (10.4%).

In the pre-diabetic group, the β -glucans, the combination of the probiotic strain and β -glucans, and the control pasta led to the highest butyrate amounts (Figure 4.13c). Composition data appeared to explain this evidence; in fact, following beta-glucans and BC³⁰+ β -glucans administration, three butyrate-producing genera were increased, i.e. *Eubacterium* (21.2% and 25.5%, respectively), *Catenibacter*, another genus including species that produce butyrate

(Kageyama and Benno, 2000) (1.9% and 3.7%, respectively) and *Roseburia* (1.7% and 2.6%, respectively). Increases in butyrate amount after treatment with the control pasta could be related to *Eubacterium* (15.6%). In conditions with high butyrate proportion (diluted functional and control pasta) (Figure 4.12) *Eubacterium* appeared increased (9.0% and 12.7%, respectively). BC³⁰+ β -glucans also lead to a high proportion of butyrate, and thus, considering the fact that they led to a high absolute amount, it represented the treatment with the best butyrate yield.

Finally, in the diabetic group, the highest butyrate production, intended as maximum average value, was obtained with the treatment with the diluted control pasta (Figure 4.13c), and it could be due explained on the basis of an increase in *Eubacterium* (5.7%), *Gemmiger* (7.3%) and *Roseburia* (5.3%), all genera known to contain butyrate producers (Louis *et al.*, 2010; Gossling and Moore, 1975; Duncan *et al.*, 2006). With this treatment also butyrate proportion was high (Figure 4.12), so the functional pasta represent the best treatment in terms of butyrate yield. Regarding proportion, high butyrate was observed also after administration of β -glucans, probably due to the increase of *Eubacterium* (7.5%) and *Roseburia* (4.2%), of the combination of BC³⁰ and β -glucans, due to the increase of *Eubacterium* (12,8%), and of the diluted control pasta, due to the increase of *Eubacterium* (3.7%), *Gemmiger* (13.5%) and *Roseburia* (3.7%).

Regarding propionate production, the highest levels were reached after treatment with the control meal, in particular in healthy and diabetic microbiota (Figure 4.13d). High propionate levels could be related to *Bacteroides* population (Macy *et al.*, 1978). In fact, this genus remained stable during treatment with the control meal, for the healthy (13% at time 0h and time 72h) and diabetic group (26% at time 0 hours and 32% at time 72 hours) (data not shown).

4.4. Concluding remarks

In this study the effects of different diets on gut microbiota from three groups of children with different susceptibility to T1D were investigated, through the use of an *in vitro* model of the colon, TIM-2. This system represents an excellent tool to investigate specific diet-microbiota interactions, through analyses of microbiota composition and produced metabolites.

Results from the composition analyses showed that a specific diet produced different responses in the three microbiota, i.e. modulations of different bacterial genera or species. However, even considering the microbiota-dependent differences in the response to a nutritional treatment, interestingly, for certain treatments, it was possible to observe increases of bacterial groups that were modulated with the same trend in the three microbiota, thus obtaining information about specific diet-microbiota relationships. For example, diets with a high concentration of starch had

a strong bifidogenic effect, or administration of the pasta enriched with the probiotic strain BC³⁰ specifically increased *Lactobacillus* populations.

Regarding metabolite profiles, highest total SCFA production was obtained with meals at highest concentrations of starch; in most cases, with these treatments, acetate was the principal produced metabolite. Treatments that led to the highest yield in butyrate, intended as both absolute amounts and proportions, were the diluted functional pasta in the healthy and diabetic microbiota, and BC³⁰+ β -glucans, in the pre-diabetic microbiota.

The merging between results of microbial composition and metabolites generally fitted well one to each other. For example, the high increase in *Bifidobacterium* populations, following treatments including the highest dose of starch, corresponded to a high production of acetate, consistently to *Bifidobacterium* metabolism.

In general, while microbiota composition analyses revealed more punctual differences in the response to a diet among the three microbiota, pointing out patterns of specific microbial groups, production of metabolites appeared to be more similar among the three microbiota; thus, a certain diet lead to a similar SCFA production in the three microbiota, despite differences in microbiota composition, probably due to functional redundancy (Louis *et al.*, 2010).

Evidences from the present study provided preliminary information about the effects of tested diets on gut microbiota; further studies will be necessary to confirm these findings, for example increasing the number of microbiota donors, also in the perspective of planning an intervention study *in humana*.

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Third part

Chapter V

Evaluation of the effects of a new functional pasta on gut microbiota of overweight and obese subjects

5.1. Introduction

Obesity represents one of the most important health issues of our time (Clarke *et al.*, 2012). According to the World Health Organization (WHO), in 2014, more than 1.9 billion adults were overweight; of these, over 600 million were obese (WHO, 2016). In percentages, this corresponds to 39% of overweight adults worldwide, and 13% of obese subjects.

Obesity is a multifactorial condition, in which modern eating habits coupled with sedentary lifestyles are the most important ones. However, other risk factors are emerging, and in particular, the involvement of gut microbiota is receiving a lot of attention (Brahe *et al.*, 2016). Studies in both animal models and *in humana* showed that there are differences in composition of microbiota between lean or obese individuals (Clarke *et al.*, 2012). At the phylum level, a lower ratio between Bacteroidetes and Firmicutes was found in obese subjects, compared to the healthy controls (Ley *et al.*, 2006; Turnbaugh *et al.*, 2009). However, following studies reported contrasting results (Schwiertz *et al.*, 2010; Duncan *et al.*, 2007), so the usefulness of this biomarker has been discussed. At lower taxonomic levels, e.g. genus or species, differences among lean and overweight/obese subjects are reported in several studies (e.g. Kong *et al.*, 2014; Schwiertz *et al.*, 2010), but it is difficult to point out common features, intended as bacterial *taxa* characteristic of the lean or obese conditions, shared among different studies; this can be due to the choice of the methodology used or to clinical factors related to the investigated subjects (Finucane *et al.*, 2014). In the study of Walters and colleagues (2014), *Faecalibacterium prausnitzii* was reported to be decreased in obese subjects analysed in several studies, thus representing a consistent biomarker.

Basing on the differences observed among lean and overweight/obese subjects, and considering the impact of the diet in shaping the gut microbiota (Graf *et al.*, 2015), intervention studies based on the use of probiotic, prebiotics or other bioactive ingredients represent a strategy to modulate

gut microbiota towards an healthy state, thus contributing to the general status of the individual (Brahe *et al.*, 2016).

Epidemiological evidences indicate that a high consumption of whole grain cereals is associate to a reduced risk of chronic disease, e.g. cardiovascular disease, type 2 diabetes, obesity (Cooper *et al.*, 2015). Mechanisms lying behind these positive effects are not clear, but the higher fibre content and the presence of phytochemical compounds that characterize whole grain products could have a role in this protective effect (Tuohy *et al.*, 2012).

Moreover, at the gut microbiota level, whole grain cereal can promote the growth of specific microbial groups. For example, whole grain cereals are able to increase *Bifidobacterium* amounts, as reported in *in humana* studies (Costabile *et al.*, 2008; Christensen *et al.*, 2013). Marked effects of high-fibre content are recently been related to species of the genus *Prevotella* (Kovatcheva-Datchary *et al.*, 2015).

β -glucans are known to have beneficial properties in lowering glucose and cholesterol levels, as recently recognized by EFSA (EFSA, 2011), thanks to their viscosity that reduce intestinal motility. At gut microbiota level, β -glucans are known to be able to increase *Bifidobacterium* and *Lactobacillus* levels, as observed from *in vitro* (Hughes *et al.* 2008; Kedia *et al.*, 2009) and *in vivo* studies (Mårtensson *et al.*, 2005; Mitsou *et al.*, 2010).

Probiotics represent another strategy for modulating gut microbiota composition and activities (Nova *et al.*, 2016). The probiotic strain *Bacillus coagulans* GBI-30, 6086 (thereafter BC³⁰) is a commercial probiotic strain reported to improve immunological parameters, intestinal functionality and physical performances (Kimmel *et al.*, 2010; Dolin, 2009; Jäger *et al.*, 2016). However, few studies are available about the impact of this probiotic strain on the gut microbiota (Honda *et al.*, 2011; Nyangale *et al.*, 2014; Nyangale *et al.*, 2015). In particular, *B. coagulans* BC³⁰ was reported to increase *F. prausnitzii* level in a human trial (Nyangale *et al.*, 2015). Moreover, it was observed to increase the level of a recently described species, *Fusicatenibacter saccharivorans* (Takada *et al.*, 2013), in another *in vitro* research (Chapter IV).

The aim of this study was to investigate the effects of a whole grain functional pasta enriched in β -glucans and in the probiotic strain *B. coagulans* BC³⁰ on the gut microbiota of overweight/obese subjects, in a 12-week parallel trial. Firstly, the overall modulation of microbiota composition following administration of the functional pasta was monitored through PCR-DGGE analyses. Then, four microbial *taxa* specifically related to the different ingredients of the functional pasta, i.e. the genera *Prevotella* and *Bifidobacterium*, and the species *Faecalibacterium prausnitzii* and *Fusicatenibacter saccharivorans*, were assessed through

qPCR; results were compared with those obtained following consumption of a whole grain control pasta, that did not include β -glucans and the probiotic strain.

5.2. Materials and Methods

Study design

The study design is a randomized, parallel, 12 week trial. Thirty-nine healthy subjects (16 males and 23 females, aged 29-74 years old, BMI > 25 kg/m²) participated to the study. Subjects were habitual consumers of pasta (≥ 70 g/day), and poorly consumers of whole grain products, fruit and vegetables. Exclusion criteria were habitual use of medications which can influence glucidic and lipidic profiles, inflammatory status, blood pressure, and intestinal functions, and use of food supplements, i.e. prebiotics and probiotics; antibiotic use in the previous three months; presence of metabolic, intestinal and immune pathologies; pregnancy and breast-feeding conditions.

Recruited subjects were assigned in a random order to one of two treatments: a) a functional pasta obtained from whole wheat flour and supplemented with β -glucans from barley and the probiotic strain *B. coagulans* BC³⁰. Subjects assigned to this treatment were named as “treated” group; b) a control pasta produced with the same technological process and from the same whole wheat flour, but without addition of β -glucans and the probiotic strain. Subjects assigned to this treatment were named as “control” group. Detailed characteristics of the functional and control pasta are reported in Chapter 1.

Subjects were asked to maintain their usual eating habits and their usual level of physical activity throughout the study, and to replace the daily portion of pasta they habitually consumed with one of the test products.

Control and functional pasta

The functional pasta, and in particular the cooked product, contained a microbial load above 6 log CFU/g of the probiotic strain *B. coagulans* BC³⁰, as previously reported (Chapter 1). Instead, the cooked control pasta did not contain bacteria of the genus *Bacillus*.

Collection of fecal samples

At the baseline of the study and every 4 weeks, for a total of 4 times, fresh fecal samples were collected at the Department of Food and Drug (University of Parma), and stored at 4°C in RNAlater (Sigma-Aldrich, Saint Louis, MO, USA). For molecular analyses, they were further subdivided in 200 mg aliquots and stored at -20°C till processing.

Microbiological analysis on fecal samples

DNA extraction procedures

Extraction of DNA from 200 mg fecal sample was performed following the protocol of QIAamp DNA stool Mini Kit (Qiagen, Hilden, Germany), as described by the manufacturer with a lysis temperature of 95°C. DNA concentration and quality were measured using a NanoDrop Lite UV-Vis Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

PCR amplification and DGGE analysis

PCR fragments representing total fecal microbiota were amplified with the universal primers HDA1-GC and HDA2 (Walter *et al.*, 2000); PCR fragments representing species of the genus *Bifidobacterium* were amplified with the primers Bif164 and Bif662 (Satokari *et al.*, 2001). The amplification products of bifidobacteria were further amplified by a nested PCR reaction using primers HDA1-GC and HDA2. PCR amplifications were conducted in the conditions reported by Fasoli *et al.* (2003).

PCR amplicons were separated by denaturing gradient gel electrophoresis (DGGE) using the D-Code Universal Mutation Detection System (Bio-Rad Laboratories, Richmond, CA, USA), basically as described previously (Fasoli *et al.*, 2003; Marzotto *et al.*, 2006). The denaturing gradients of urea and formamide used for discrimination of amplicons from total microbiota and bifidobacteria were 30–60% and 45–60%, respectively. The polyacrylamide gels were run at 60°C in 1 × TAE buffer at 90 V for 10 min, and then at 50 V for 16 h. After electrophoresis, gels were stained with EuroSafe Nucleic Acid Stain (Euroclone, Milan, Italy) for 15 min, washed and photographed under UV illumination.

DGGE ladders for the electrophoretic runs were prepared by mixing equal amounts of amplicons obtained from selected reference species using primers HDA1-GC and HDA2, as previously described (Marzotto *et al.*, 2006).

DGGE patterns were analysed with BioNumerics software (Version 7.5; Applied Maths, Sint-Martens-Latem, Belgium). Similarities between DGGE profiles were determined using the Dice coefficient, and the unweighted pair group method with the arithmetic average (UPGMA) was used to construct the corresponding dendrogram.

Quantitative PCR analyses

Quantitative real-time PCR (qPCR) assays were used for the detection of *Prevotella* spp., *Bifidobacterium* spp., *Faecalibacterium prausnitzii* and *Fusicatenibacter saccharivorans* in fecal

samples. Enumeration of species and genera mentioned above was conducted using the primers reported in Table 5.1.

Quantitative PCR assays were performed with a LightCycler Nano System (Roche, Mannheim, Germany), using the FastStart DNA Master SYBR Green kit (Roche, Mannheim, Germany), in a final volume of 20 μ L, containing 5 μ L of each fecal DNA preparation, 0.25 μ M of each primer, MasterMix buffer 1 \times and PCR grade water. No-template controls were included in each assay. The thermal cycling conditions used for each species or genera analysed consisted in one cycle of 95°C for 10 min, followed by 45 cycles with denaturation at 95°C for 15 s, primer annealing at the temperature reported in Table 5.1 (T_a) for 15 seconds, and extension at 72°C for the time reported in Table 5.1.

Standard curves were constructed using 10-fold serial dilutions of genomic DNA of known concentration from the reference strains (i.e. *Prevotella copri* DSM 18205^T, *Bifidobacterium adolescentis* LMG 11037^T, *Faecalibacterium prausnitzii* DSM A2-165^T and *Fusicatenibacter saccharivorans* DSM HT03-11^T). For each dilution, the genomic copies were plotted against the cycle number at which the fluorescence signal increased above the threshold value (C_T value). For all primer pairs, qPCR amplification efficiency was obtained from the equation $E=10^{(-1/\text{slope})}$. Cell numbers of bacteria in fecal samples were calculated by comparing the threshold cycle values with the standard curve, and expressed as CFU per gram of feces. All PCR analyses were carried out in technical triplicate, and presented data are the mean values obtained.

Table 5.1. Primer sequences and specifications for qPCR assays (annealing temperature and extension time).

Target	Primer	Sequence (5'-3')	Length (bp)	T_a (°C)	Extension time (sec)	Reference
<i>Prevotella</i> spp.	PrevF PrevR	CACCAAGGCGACGATCA GGATAACGCCYGGACCT	283	62	12	Larsen <i>et al.</i> , 2010
<i>Bifidobacterium</i> spp.	Bif164 Bif662	GGGTGGTAATACCGGATG CCACCGTTACACCGGGAA	520	63	20	Satokari <i>et al.</i> , 2001
<i>Faecalibacterium prausnitzii</i>	FPR-2F Fprau645R	GGAGGAAGAAGGTCTTCGG AATTCCGCCTACCTCTGCACT	248	60	11	Ramírez-Farias <i>et al.</i> , 2009
<i>Fusicatenibacter saccharivorans</i>	s-Fsac-F s-Fsac-R	CTGCATTGGAAACTGTCTGG CGTTACGGGCCCGGTCATC	389	59	16	Kurakawa <i>et al.</i> , 2015

Clinical data

Anthropometric and blood analyses (e.g. glucose metabolism, inflammatory status) were performed at the Department of Clinical and Experimental Medicine, University of Parma.

Statistical analysis

SPSS was used to compare qPCR microbiological results at time 0 months and time 3 months, for each microbial *taxa* of interest, and to compare microbiological and clinical results. Statistical analyses were performed at the Department of Food and Drug, University of Parma.

5.3. Results and discussion

Assessment of modulation of resident gut microbiota through PCR-DGGE analyses

PCR-DGGE analyses were performed on the subjects who ingested the functional pasta, in order to reveal modulations of the gut microbiota due to this product, if any.

Preliminary analyses of total microbiota, conducted with universal primers HDA1-GC and HDA2, were performed for three subjects (2, 5, 13) at the different times of the clinical trial, i.e. at the beginning of the intervention period, and after 1, 2 and 3 months (T0, T1, T2 and T3). Moreover, analyses on the same subjects were performed with primer Bif164 and Bif662, targeting *Bifidobacterium* species. The aim of these analyses was to assess the stability of the gut microbiota during the whole duration of the treatment with the functional pasta.

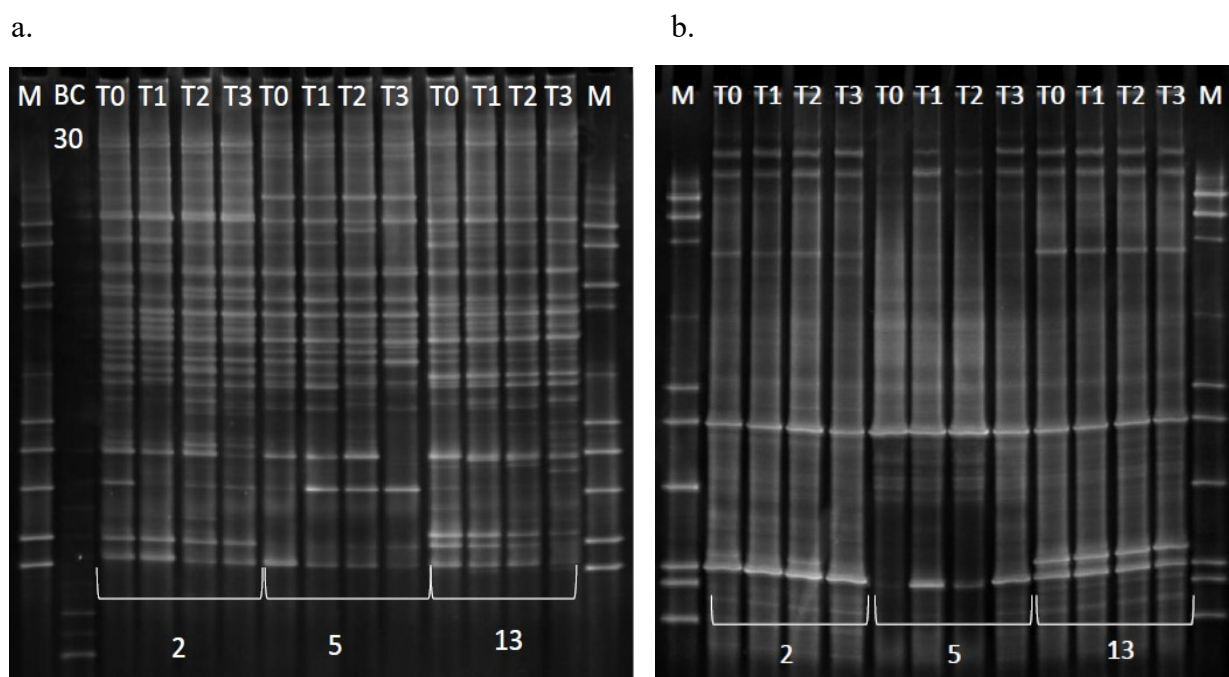


Figure 5.1. Profiles obtained from PCR-DGGE analyses of a) the total microbiota and b) *Bifidobacterium* population, for 3 subjects (2, 5, 13) who ingested the functional pasta, at the beginning (T0) and after 1, 2 and 3 months (T1, T2, T3).

Profiles from total microbiota (Figure 5.1a) appeared particularly complex, and substantially stable; in fact, few bands were observed to appear or disappear during time for any subject. In

order to assess stability of the profiles also for a subpopulation, PCR products derived from *Bifidobacterium* spp. were analysed. In particular this microbial group was analysed since it was expected to be modulated following the intervention with the whole grain product, further enriched with β -glucans. Results showed considerably simpler profiles (Figure 5.1b), and confirmed the stability observed from the analyses of the total microbiota.

Basing on this findings, following analyses were performed on the overall group of subjects who ingested the functional pasta focusing on the beginning (T0) and the end (T3) of the intervention period. Both the total microbiota population and the *Bifidobacterium* spp. were analysed at these two times. Regarding total microbiota, subjects exhibited very rich and complex profiles (Figure 5.2). The average number of the bands observed at T3 (18.8 ± 2.3) did not differed significantly from that observed at T0 (20.2 ± 2.5), according to previous findings (Abell *et al.*, 2008).

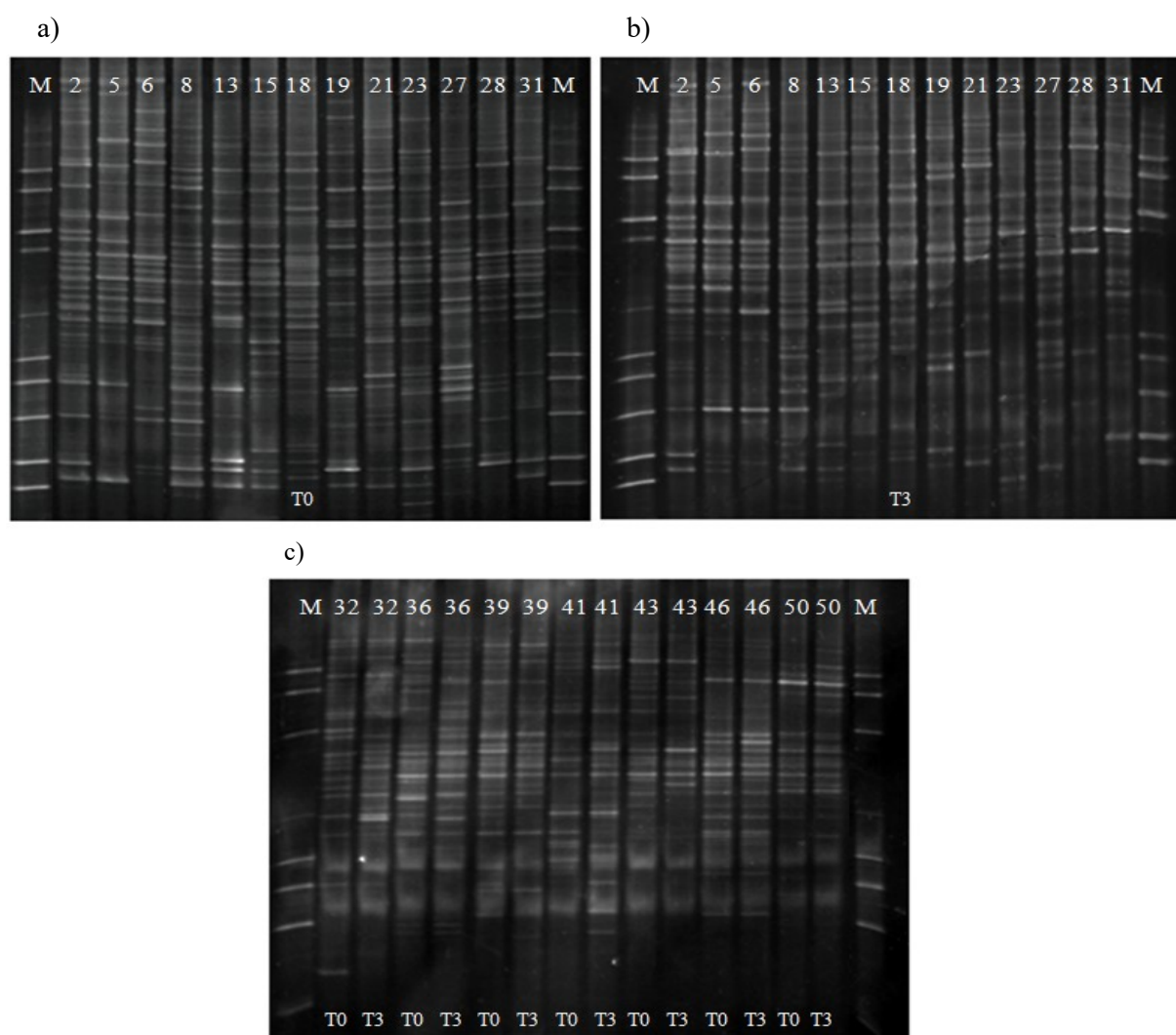


Figure 5.2. Profiles obtained from PCR-DGGE analyses of the total microbiota for the 20 subjects who ingested the functional pasta, at the beginning (T0) and at the end (T3) of the clinical trial. a) subjects 2 to 31, T0; b) subjects 2 to 31, T3; c) subjects 32 to 50, T0 and T3.

Punctual differences were observed in terms of bands that appeared or disappeared in different individuals from the beginning to the end of the intervention period with the functional pasta; however, common bands that could indicate a specific effect of the functional product were not retrieved among the overall group of subjects in the T3 samples (Figure 5.2).

An inter-individual variability in the profiles of the subjects was observed, both at T0 and at T3. Moreover, the patterns of the total microbiota profiles at the beginning and at the end of the trial was maintained for each individual (Figure 5.2). This was confirmed also from the similarity analysis of the profiles (Figure 5.3). In fact, as revealed from the graph, profiles mostly clustered basing on individuals (16/20) and not on the sampling point, showing that inter-individual differences are stronger than the effects of the treatment. Few subjects represented an exception: profiles from subjects 19, 21 and 28 resulted intermixed with those from other subjects; profiles from subject 13 resulted in a subcluster with profiles from subject 39. The fact that differences among individuals can be greater than differences observed for a certain individual between different analysis times following a certain dietary intervention was previously observed (Abell *et al.*, 2008, Walker *et al.*, 2011).

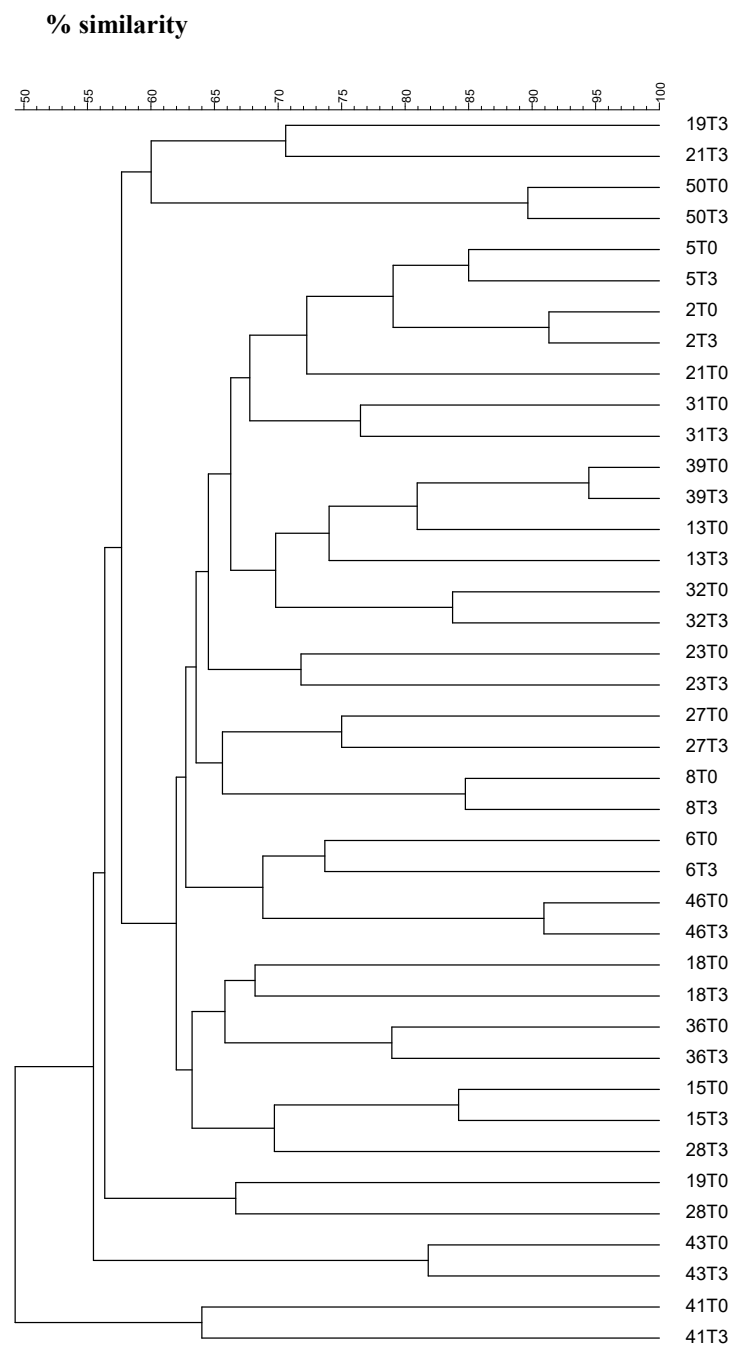


Figure 5.3. Dendrogram obtained from the profiles of the total microbiota for the 20 subjects who ingested the functional pasta, at the beginning (T0) and at the end (T3) of the clinical trial.

Analyses of *Bifidobacterium* population showed less complex profiles, with a low average band number ($T0 = 5.35 \pm 2.2$; $T3 = 5.85 \pm 1.7$) than that observed for the total microbiota (Figure 5.4). Subjects exhibited, again, a certain inter-individual variability, even if, respect to profiles obtained for total microbiota, patterns are more similar among individuals, due to the lower number of bands obtained. Stability in the profiles was observed for most individuals during time, as also reported previously (Salazar *et al.*, 2015); some specific subjects (e.g. 6, 39) exhibited more marked differences in terms of bands between the beginning and the end of the intervention period. However, no bands differentially present at the beginning or at the end of the treatment and shared among the overall group of subjects were detected. As shown from the similarity analyses, profiles clustered according to the different individuals, and not according to time, T0 or T3 (Figure 5.5).

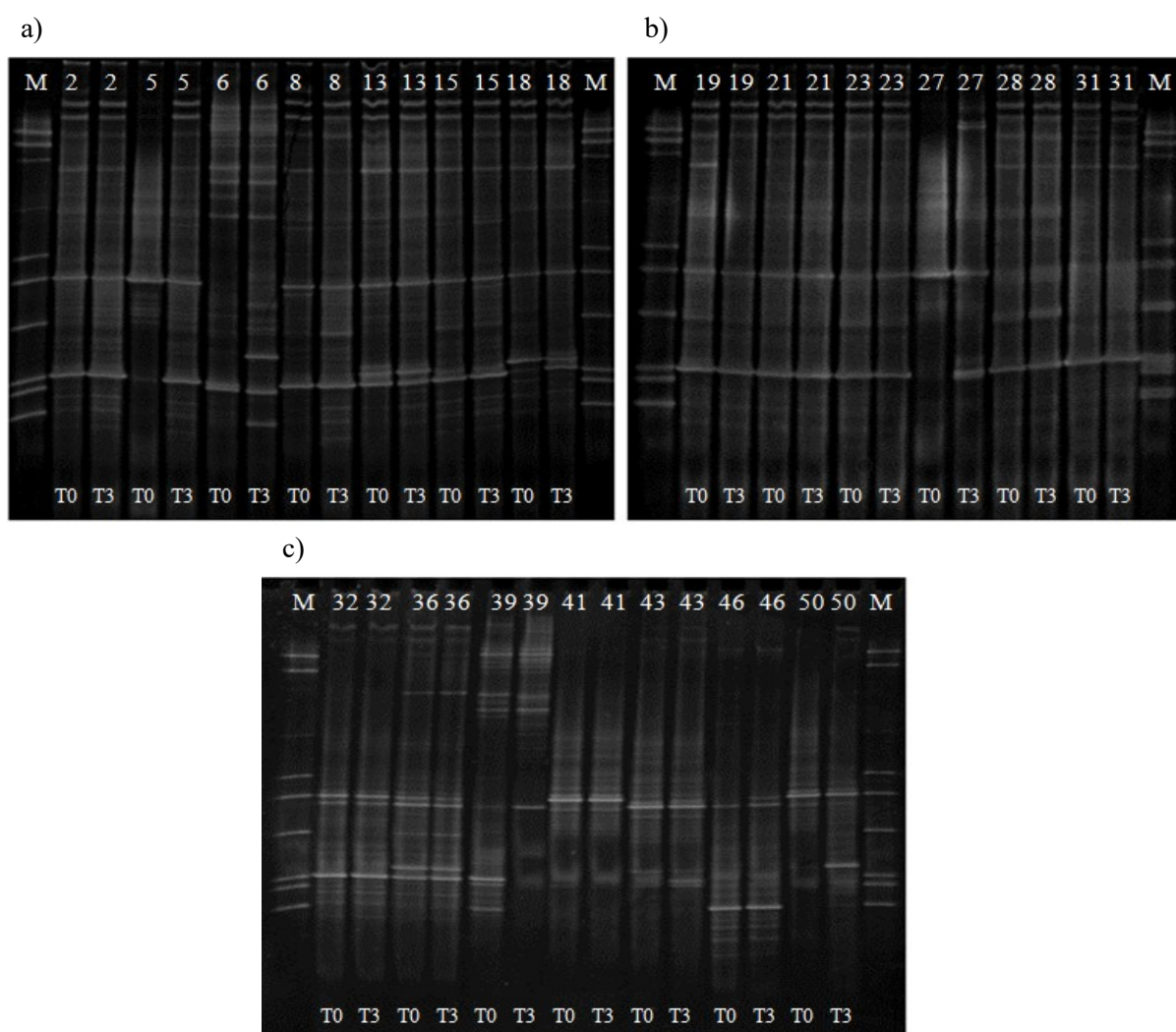


Figure 5.4. Profiles obtained from PCR-DGGE analyses of *Bifidobacterium* population for the 20 subjects who ingested the functional pasta, at the beginning (T0) and at the end (T3) of the clinical trial. a) subjects 2 to 18, T0 and T3; b) subjects 19 to 31, T0 and T3; c) subjects 32 to 50, T0 and T3.

% similarity

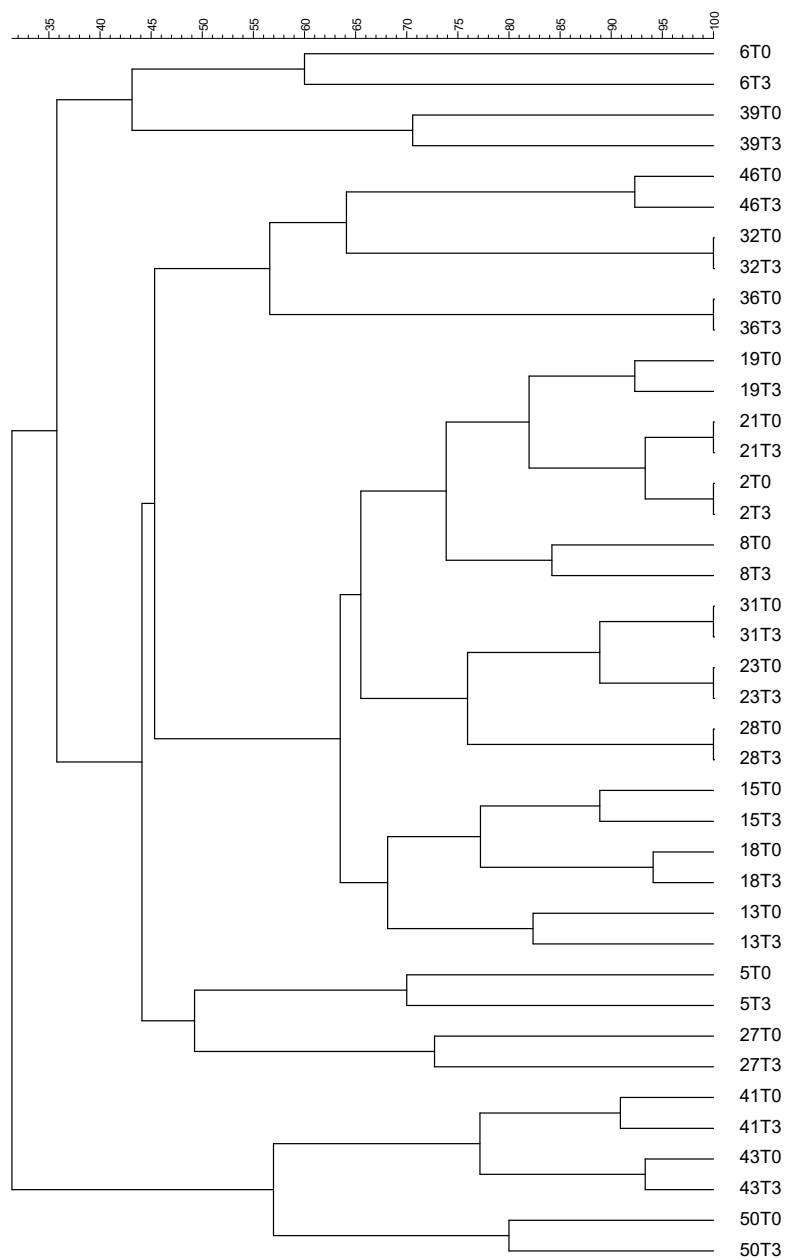


Figure 5.5. Dendrogram obtained from the profiles of the *Bifidobacterium* population for the 20 subjects who ingested the functional pasta, at the beginning (T0) and at the end (T3) of the clinical trial.

Investigation of specific microbial *taxa* through qPCR

Considering the substantial stability in the microbiota profiles of the subjects before and at the end of the intervention with the functional pasta, observed through PCR-DGGE, qPCR analyses were performed in order to investigate quantitative changes of specific microbial groups, expected to be modulated from the different ingredients of the functional pasta. Thus, the genera *Prevotella* and *Bifidobacterium*, and the species *Faecalibacterium prausnitzii* and *Fusicatenibacter saccharivorans* species were investigated. In this case qPCR analyses were performed also on the subjects who were assigned to the treatment with the control pasta.

Quantifications of the genera *Prevotella* and *Bifidobacterium* and the species *F. prausnitzii* and *F. saccharivorans* were performed through the use of standard curves presenting the equations: $y = -3.67x + 36.49$, $y = -3.98x + 42.67$; $y = -3.68x + 37.31$, $y = -3.40x + 29.95$, respectively. Efficiency with the used primer pairs ranged from 1.78 to 1.97. The limit of detection were 10^4 CFU/g of fecal sample with respect to genus *Bifidobacterium* and *F. prausnitzii*, and 10^3 CFU/g of fecal sample regarding genus *Prevotella* and *F. saccharivorans*.

The genus *Prevotella* was selected as it is reported to be associated with a Mediterranean diet, based on a large consumption of cereals, fruits and vegetables (De Filippis *et al.*, 2015). In addition, studies aimed at assessing the effects of fiber content in foods showed increases in this microbial genus (Kovatcheva-Datchary *et al.*, 2015; Vitaglione *et al.*, 2015).

For the analysis of such genus, the 39 subjects of the *trial* were combined into one large group, because of the fact that the amount of total fibre contained in the cooked pasta, functional and control, is comparable for the two products (Chapter 1).

At the end of the clinical trial, increases or decreases of the genus *Prevotella* were observed, for different subjects, with a more or less marked trend (Figure 5.6).

Considering the value of one logarithm as a threshold for defining a relevant modulation, five (2, 9, 19, 24, 38) out of the 39 subjects exhibited such an increase, while a decrease was observed in two subjects (23, 51). Among the subjects with an increase in *Prevotella*, subject 19 exhibited a two logarithm increase, while a two logarithm decrease was observed for subject 51. However, considering the whole group of subjects, there were no statistically significant changes between the quantities of *Prevotella* measured at the beginning and at the end of the trial (Figure 5.7).

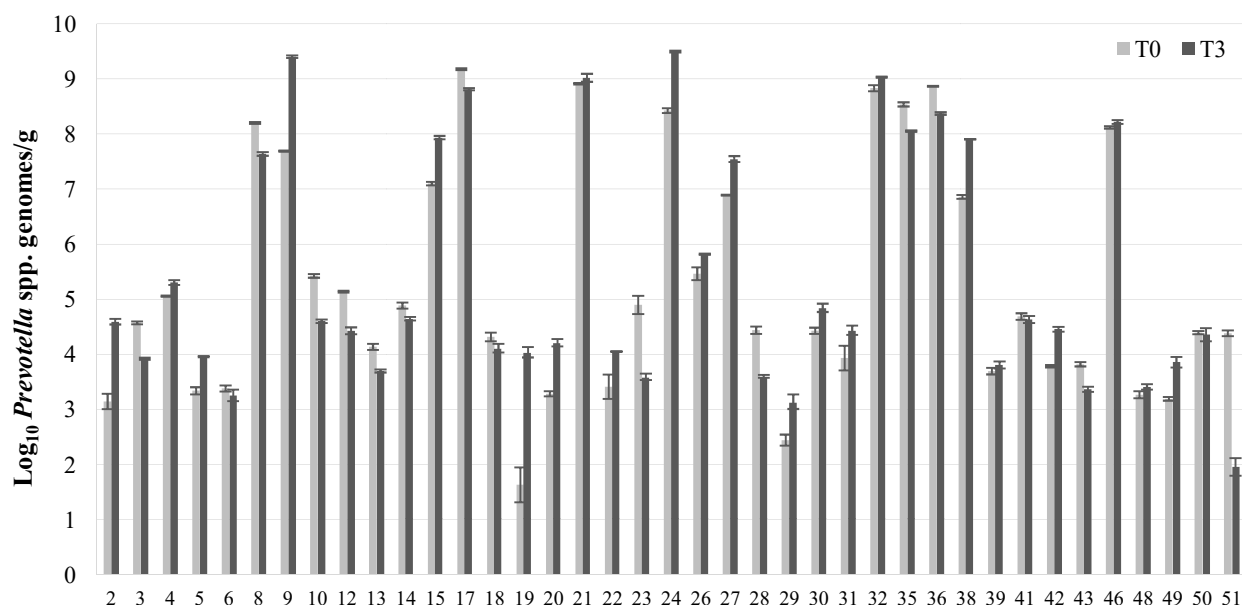


Figure 5.6. Quantification of *Prevotella* spp. in all subjects, at the beginning (T0) and at the end (T3) of the intervention period.

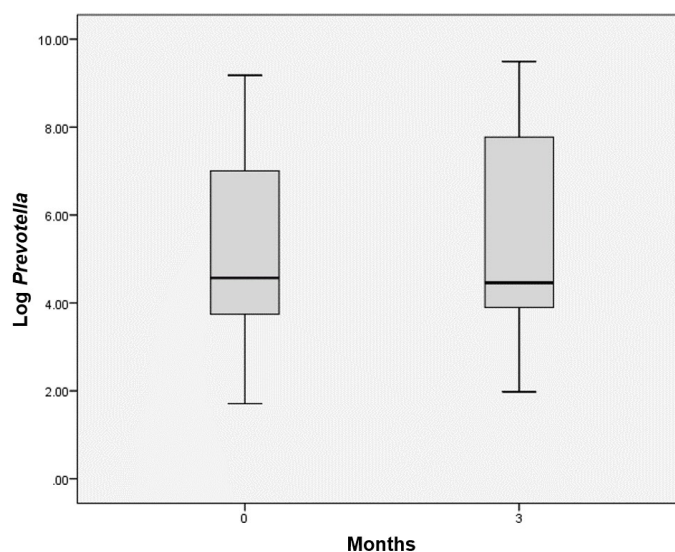


Figure 5.7. Box-plot representation of the amounts of *Prevotella* spp. in all subjects, at the beginning (month 0) and at the end (month 3) of the intervention period.

Subjects were then stratified according to their BMI, and divided into two groups, overweight (BMI ≤ 29.9 kg/m²) and obese (BMI ≥ 30.0 kg/m²), the first consisting in 23 overweight subjects, and the second consisting in 16 obese subjects (Figure 5.8).

Even with this stratification, there were no significant variations in the quantities of *Prevotella*, at the beginning and end of treatment (Figure 5.9), although an higher median value in *Prevotella* levels was observed in the group of the obese subjects, after 12-week treatment with the pasta.

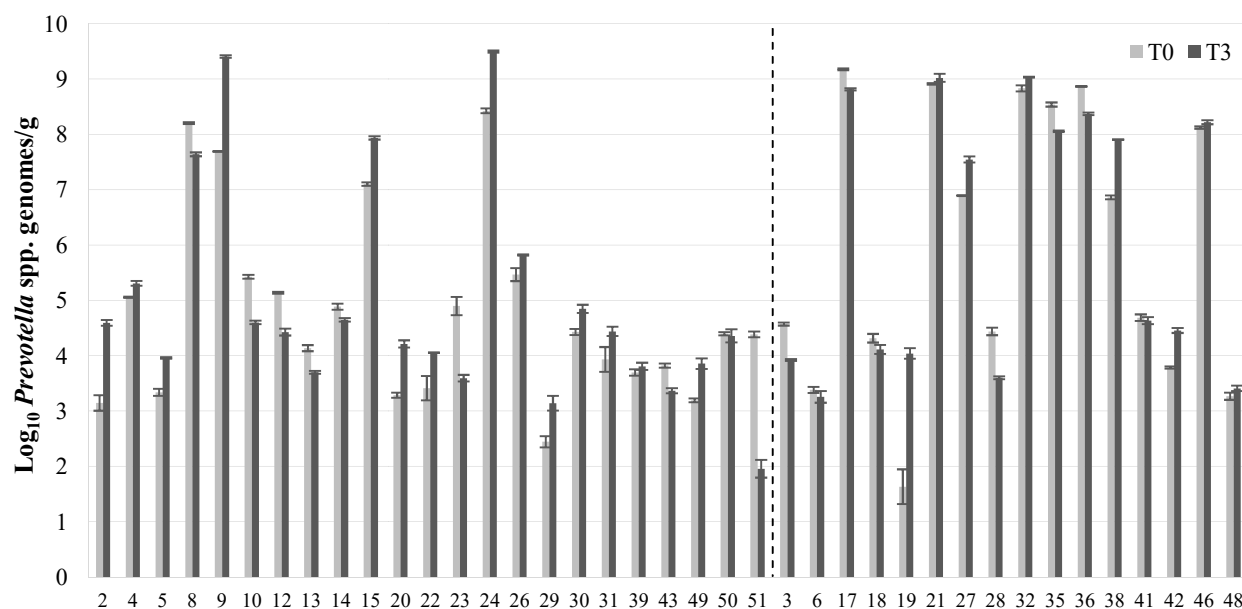


Figure 5.8. Quantification of *Prevotella* spp. in the overweight (left) and obese (right) groups of subjects, at the beginning (T0) and at the end (T3) of the intervention period.

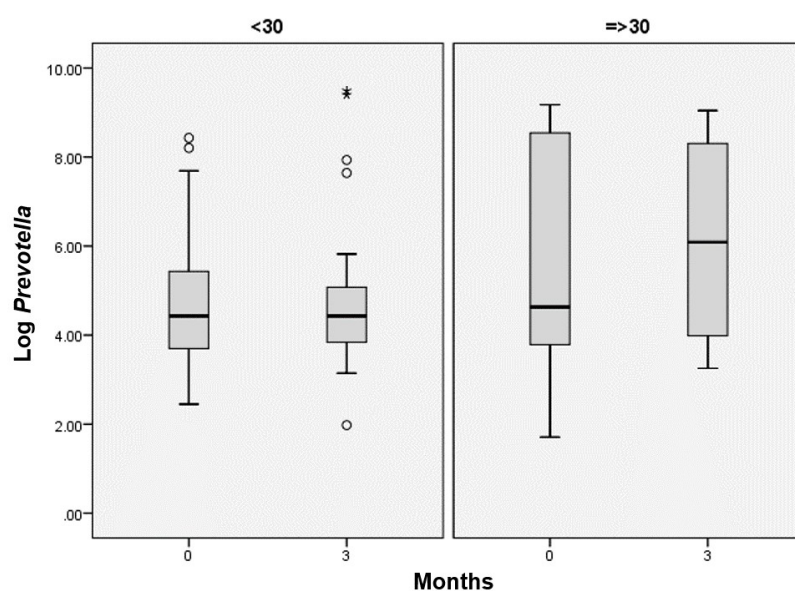


Figure 5.9. Box-plot representation of the amounts of *Prevotella* spp. in the overweight and obese groups of subjects, at the beginning (month 0) and at the end (month 3) of the intervention period.

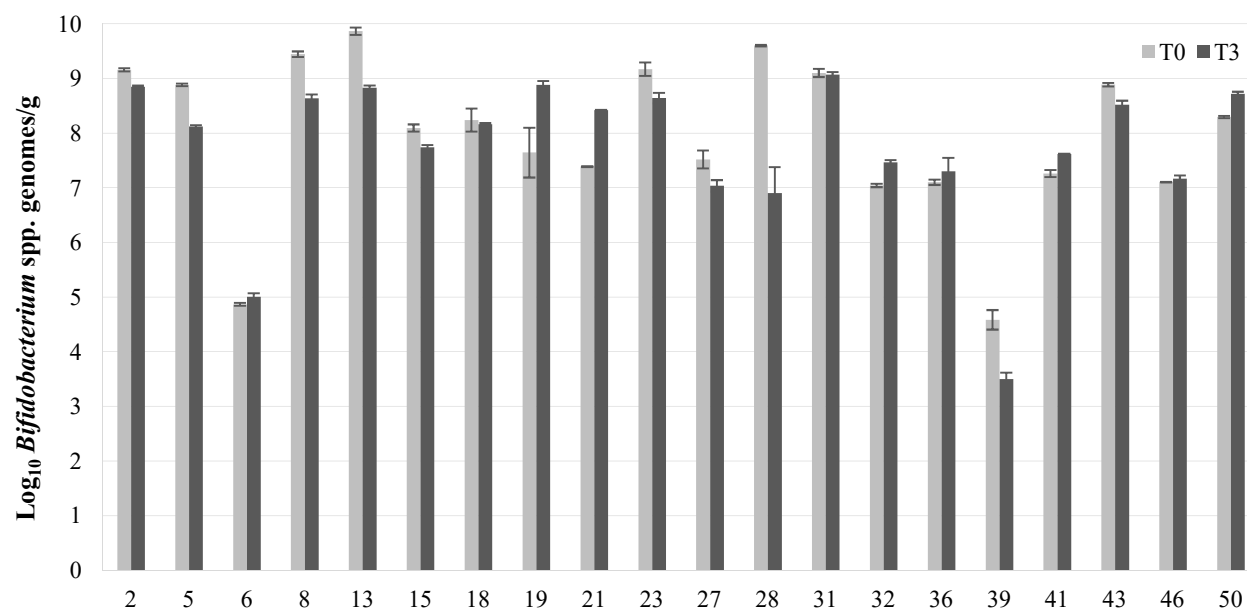
In conclusion, the results obtained by qPCR analysis showed a great variability in the modulation of *Prevotella* among subjects after consumption of the functional or control pasta, together with a pronounced variability observed at the baseline of the intervention. Following the treatment, increases or decreases in *Prevotella* levels depended on the subjects. The fact that individuals can be more or less responsive to a fibre containing treatment was also reported by Kovatcheva-Datchary and colleagues (2015); in this study, the authors showed how the consumption of the test product increases *Prevotella* levels only in some of the participants. In particular, the product improved glucose tolerance in specific individuals (subjects defined *responders*), which also showed an increase in the abundance of *Prevotella*, and specifically, in the species *Prevotella copri* (Kovatcheva-Datchary *et al.*, 2015). In the study of Vitaglione and colleagues (2015), *Prevotella* levels significantly increased after administration of whole grain products. Our results indicated a trend in *Prevotella* increase after the consumption of the products in the obese subjects.

The genus *Bifidobacterium* was selected as a second group of interest for the analysis, on the basis of several evidences showing that the consumption of whole grain foods, and/or enriched in β -glucans, increased abundance of bifidobacteria in the gastrointestinal tract (Costabile *et al.*, 2008; Christensen *et al.*, 2013; Mitsou *et al.*, 2010; Mårtensson *et al.*, 2005).

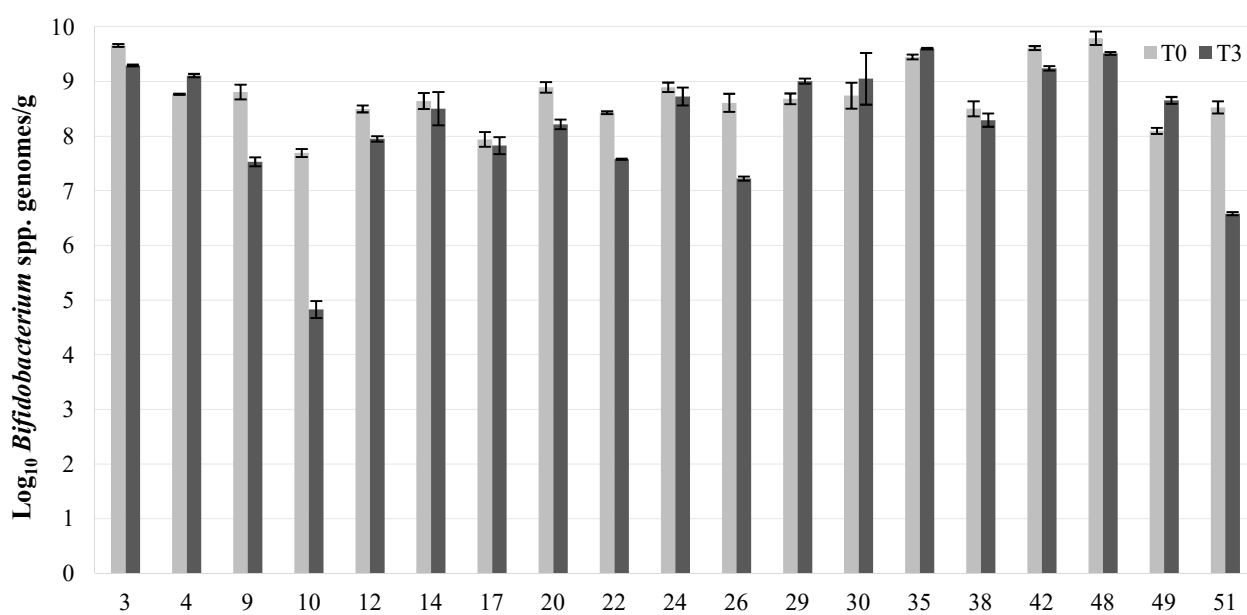
For the analysis of the genus *Bifidobacterium*, the two groups of subjects, those assigned to the functional pasta and those assigned to the control pasta, were considered separately. In fact, even if the ingestion of both types of pasta, both whole grain products, could lead to an increase of bifidobacteria, added β -glucans could further strengthen this bifidogenic effect.

Also for these microorganisms, analyses showed a varied response among subjects; for some of them an increase in *Bifidobacterium* was shown, and for others a decrease (Figure 5.10a, b). Regarding subjects who ingested the functional pasta, the threshold value of one logarithm, indicative of a relevant modulation, was observed for only two subjects, 19 and 21, that exhibited an increase in this genus. Decreases were observed for three subjects, 13, 28 and 39; subject 28 showed a two log unit decrease (Figure 5.10a).

For subjects belonging to the control group, no relevant increases were observed, while relevant decreases were observed in four subjects (9, 10, 26, 51). Subjects 10 and 51 exhibited decreases greater and around 2 log, respectively (Figure 5.10b). Statistical analysis on the whole groups of subjects, treated and control (Figure 5.11) did not show significant differences between the amounts in bifidobacteria, at the beginning and end of treatment, for both groups.



(a)



(b)

Figure 5.10. Quantification of *Bifidobacterium* spp. in the treated (a) and control (b) groups of subjects, at the beginning (T0) and at the end (T3) of the intervention period.

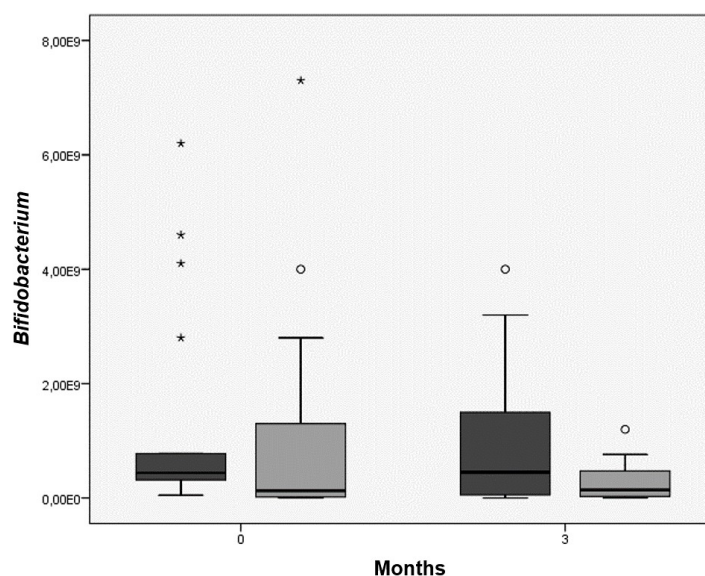
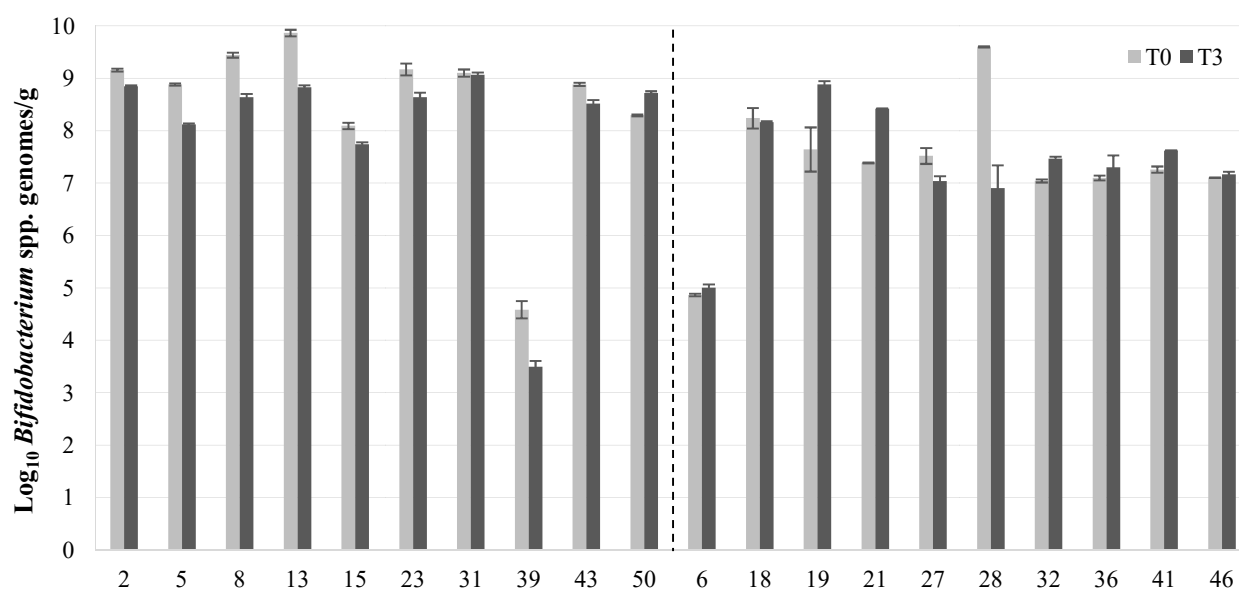


Figure 5.11. Box-plot representation of the amounts of *Bifidobacterium* spp. at the beginning (month 0) and at the end (month 3) of the intervention period. Treated group is represented in light grey, control group is represented in dark grey.

The treated group and the control group were subsequently divided into overweight and obese subgroups, according to the BMI of the subjects. The treated group included 10 overweight subjects and 10 obese subjects. In the control group, 13 subjects constituted the overweight group and 6 subjects constituted the obese one (Figure 5.12a, b).



(a)

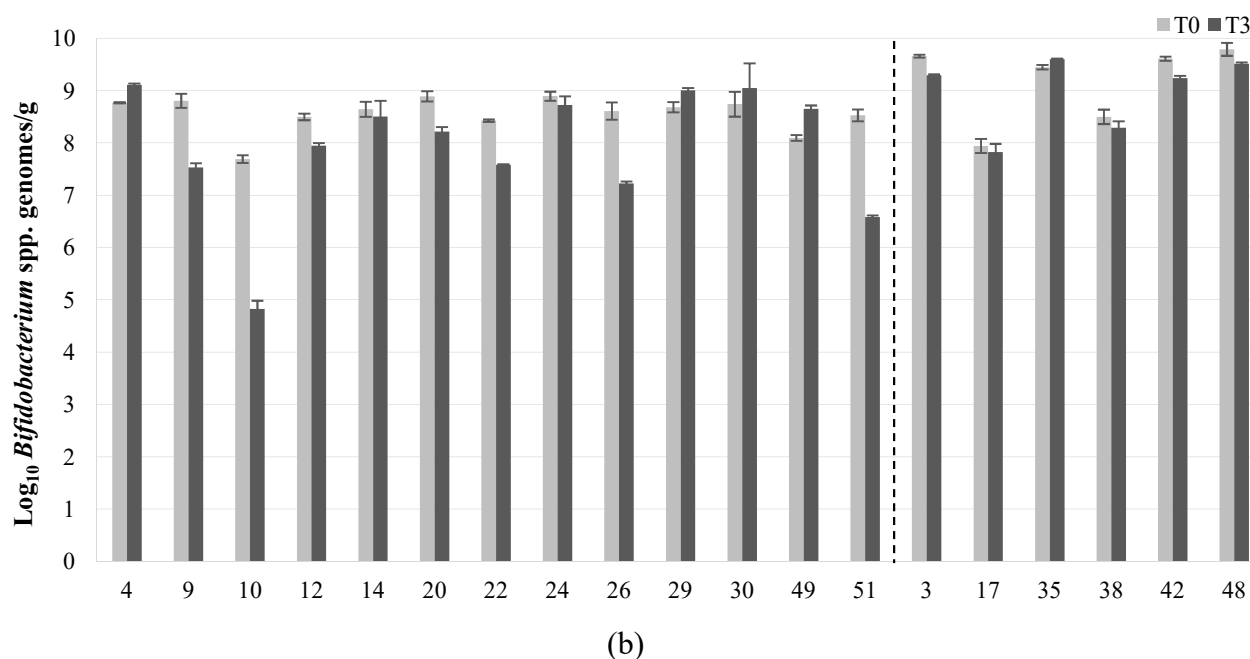


Figure 5.12. Quantification of *Bifidobacterium* spp. in the treated (a) and control (b) groups of subjects, at the beginning (T0) and at the end (T3) of the intervention period. Subjects are stratified according to their BMI, in overweight (left) and obese (right).

As a result of this stratification, in the overweight subjects who consumed the functional pasta (light grey), a significant decrease in the population of *Bifidobacterium* spp. was observed during time (Figure 5.13; ¥: $p < 0.05$, Wilcoxon Test). For the control group (dark grey), stratified in overweight and obese subjects, no significant differences at the beginning and at the end of the treatment were observed, for any of the two groups (Figure 5.13).

An interesting result emerged from the inter-group comparison, among treated and control groups, with reference to the subgroups of obese subjects. The amount of bifidobacteria in the obese control subjects (Figure 5.13) was statistically higher than the amount of bifidobacteria in the obese treated subjects, both at the beginning and at the end of the trial (#: $p < 0.05$, Mann-Whitney test).

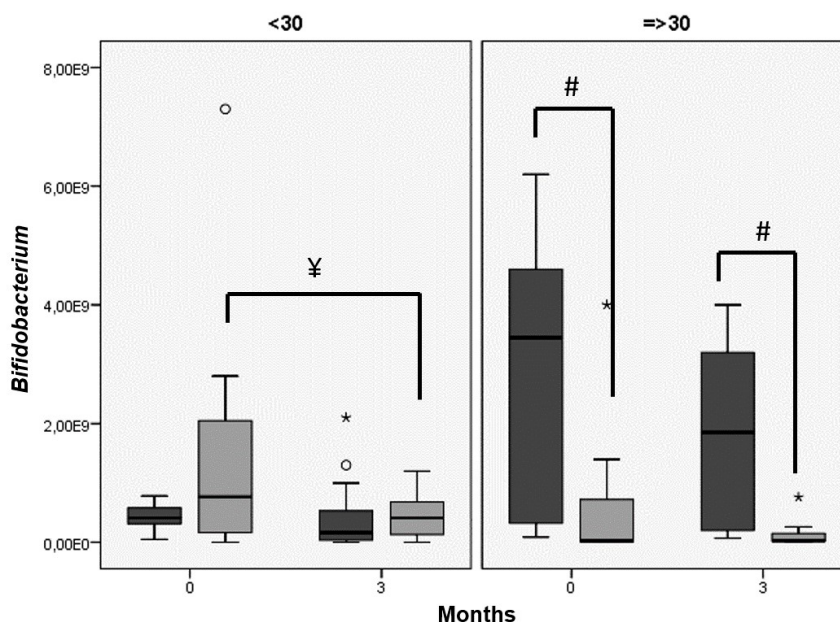


Figure 5.13. Box-plot representation of the amounts of *Bifidobacterium* spp. at the beginning (month 0) and at the end (month 3) of the intervention period. Treated group is represented in light grey, control group is represented in dark grey. Subjects are stratified according to their BMI, in overweight (left) and obese (right).

Thus, differently to what reported in many studies in literature, the ingestion of the two whole grain pasta, and furthermore, of the functional pasta with added β -glucans, did not cause an increase of the genus *Bifidobacterium*. On the contrary, stratifying subjects according to their BMI, it was observed a statistically significant decrease of bifidobacteria in the subjects treated with the functional pasta. A decrease in *Bifidobacterium* spp. following the consumption of prebiotics has been reported in a study that monitored the modulation of the microbiota in mice, following ingestion of prebiotics for a prolonged period (Li *et al.*, 2015). In that study, the authors proposed that the continuous consumption of one type of prebiotic, FOS or inulin in the specific case, for a period longer than four-weeks, did not promotes the growth of *Bifidobacterium* species anymore; moreover, it can even lead to a repression of present bifidobacteria, probably due to the development and competition with other microorganisms (Li *et al.*, 2015). Although that study was conducted in an animal model, and the type of prebiotic administered was different, a similar mechanism could explain the effects observed in the present clinical trial.

Moreover, although most studies investigating β -glucans reported a bifidogenic effect, there are some works showing contrasting results. For example, the study performed by De Angelis and colleagues (2015), which assessed the effects of a pasta enriched in β -glucans in a group of

normal weight subjects, did not report modulations related to the genus *Bifidobacterium*. In another study, examining the effects of a high fibre rye bread on the microbiota, bifidobacteria were not modulated from the intervention (Gråsten *et al.*, 2007).

Analyses of the species *Faecalibacterium prausnitzii* showed again a inter-individual variability in response to the treatments with the functional and control pasta (Figure 5.14a, b).

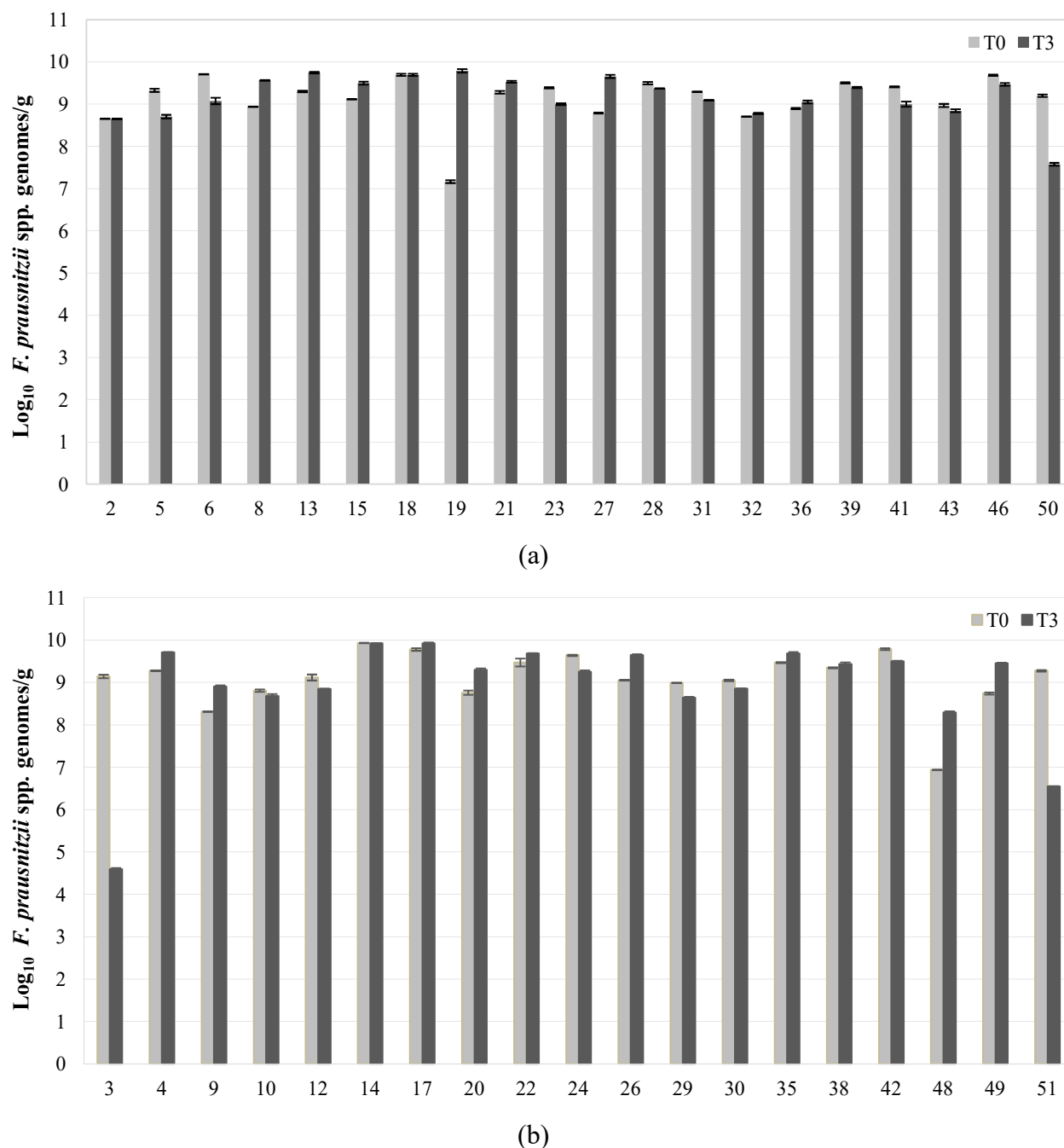


Figure 5.14. Quantification of *F. prausnitzii* in the treated (a) and control (b) groups of subjects, at the beginning (T0) and at the end (T3) of the intervention period.

Regarding analyses of the treated group, only for one volunteer, subject 19, a marked increase of two log units was observed, while a decrease of one log unit was observed for subject 50 (Figure 5.14a). For the control group, a one log increase was observed for subject 48, and a decrease of more than 2 log was observed in two subjects, 3 and 51 (Figure 5.14b). Regarding the overall groups of subjects, control and treated, no statistically significant differences in the amounts of *F. prausnitzii* between the beginning and the end of the treatment were observed (Figure 5.15).

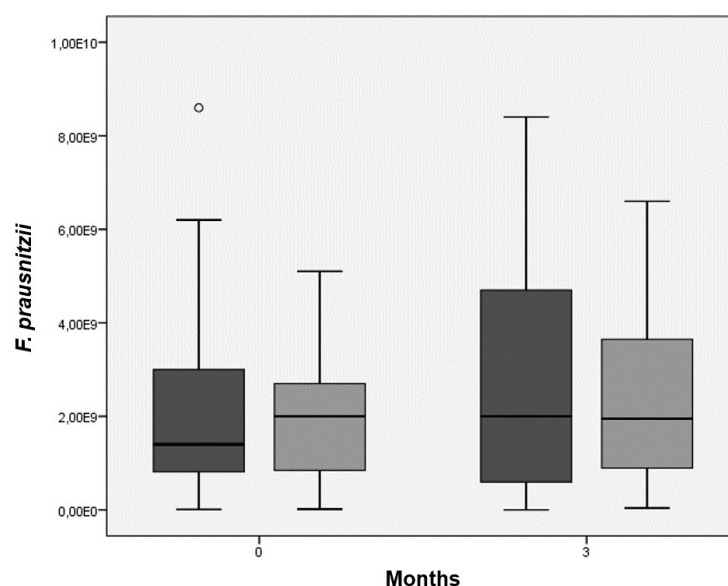
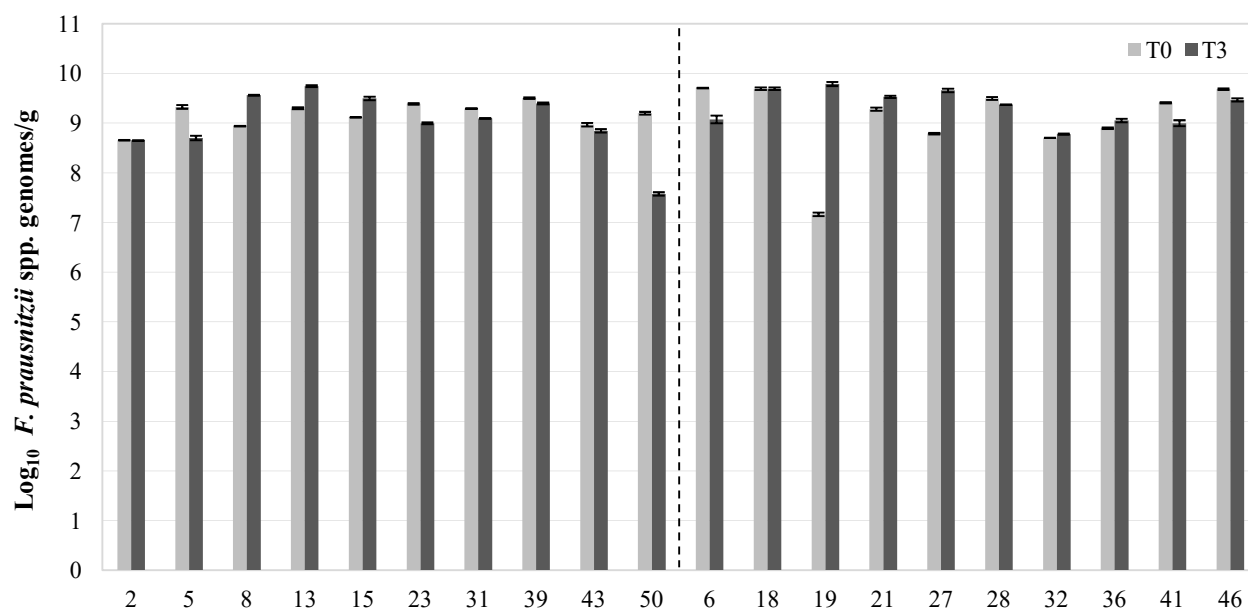
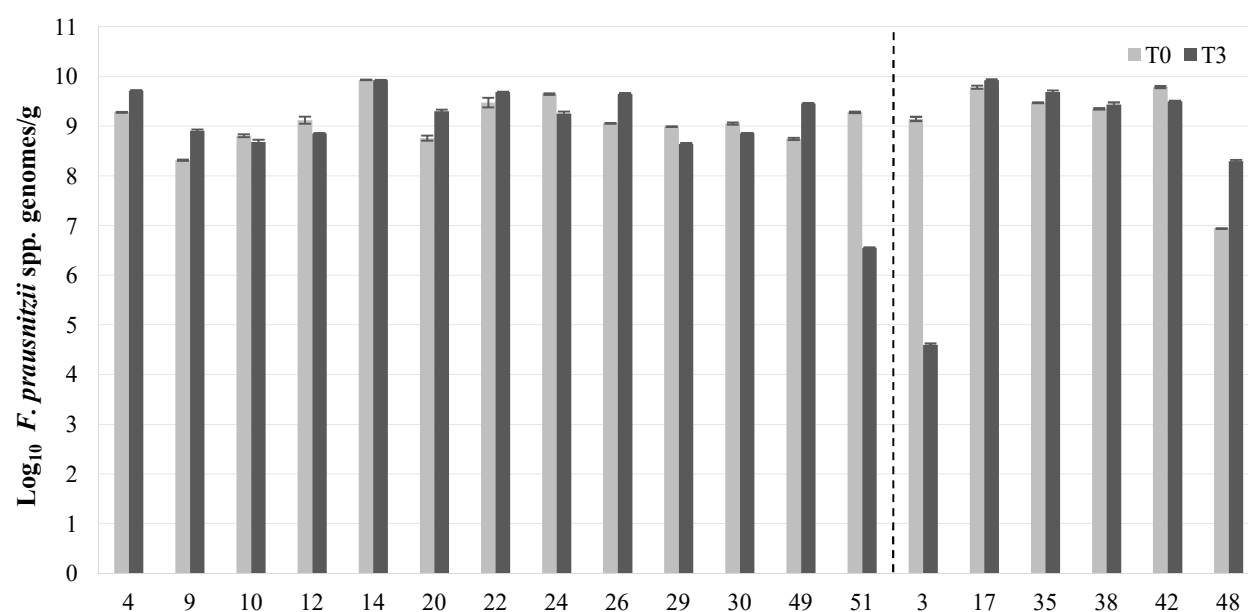


Figure 5.15. Box-plot representation of the amounts of *F. prausnitzii* at the beginning (month 0) and at the end (month 3) of the intervention period. Treated group is represented in light grey, control group is represented in dark grey.

Even stratifying the subjects in subgroups, according to their BMI, no significant modulations emerged (Figure 5.16a, b; Figure 5.17). Finally, no results were found from the inter-group comparison, treated and control, stratified or not.



(a)



(b)

Figure 5.16. Quantification of *F. prausnitzii* in the treated (a) and control (b) groups of subjects, at the beginning (T0) and at the end (T3) of the intervention period. Subjects are stratified according to their BMI, in overweight (left) and obese (right).

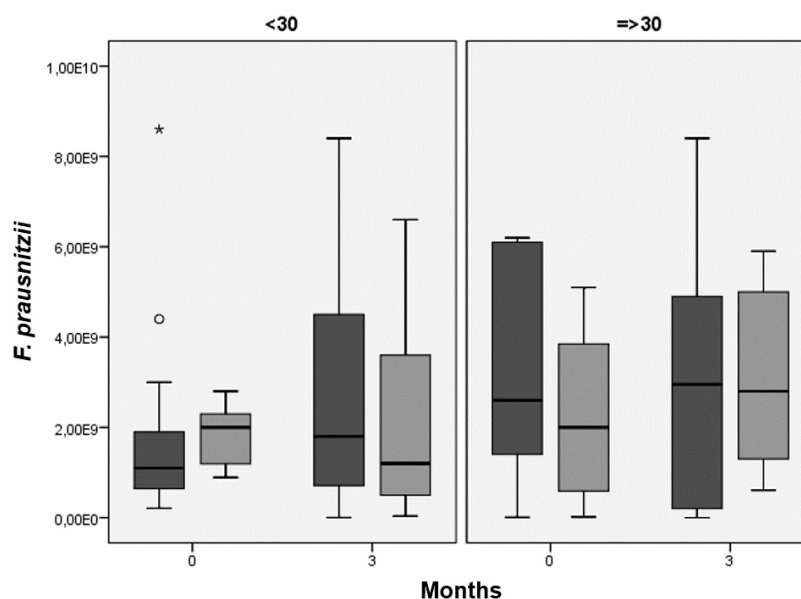


Figure 5.17. Box-plot representation of the amounts of *F. prausnitzii* at the beginning (month 0) and at the end (month 3) of the intervention period. Treated group is represented in light grey, control group is represented in dark grey. Subjects are stratified according to their BMI, in overweight (left) and obese (right).

F. prausnitzii has been investigated because it is assumed to be stimulated from the ingestion of the probiotic strain *B. coagulans* BC³⁰, as observed both *in vitro* and in a clinical trial (Nyangale *et al.*, 2014; Nyangale *et al.*, 2015). Obtained results were contrasting with these previous evidences; in fact, in the present study, a statistically significant increase in this species following consumption of the functional pasta containing the probiotic strain was not observed. This could be attributed to different factors. Firstly, subjects analysed in the work of Nyangale and colleagues had different characteristics than the subjects of the present clinical trial; in particular, they had normal weight, which can probably lead to a different response to the treatment. In addition, in the mentioned trial, which is the only one available in literature dealing with the effects of the consumption of the probiotic strain on the gut microbiota, *B. coagulans* BC³⁰ was administered as a supplement, and not included in a food with other functional ingredients. In the present study, the effect of β -glucans in combination with fibre could have reduced the stimulation of *F. prausnitzii*. In fact, the consumption of a pasta enriched in β -glucans lead to a significant decrease in the levels of *F. prausnitzii* in a recent clinical trial (De Angelis *et al.*, 2015).

Finally, the effects of the functional and control pasta were investigate with reference to the species *Fusicatenibacter saccharivorans*. Few information about this species is available. It

resulted decreased in hospitalized children suffering from a chronic inflammatory disease (enthesitis-related arthritis, ERA) together with a decrease in *F. prausnitzii*; these findings lead to the hypothesis that these reductions may be related to the onset of the disease condition (Stoll *et al.*, 2014). In the present work, *F. saccharivorans* was investigated since it was found that the probiotic strain BC³⁰ could promote its growth (Chapter 4).

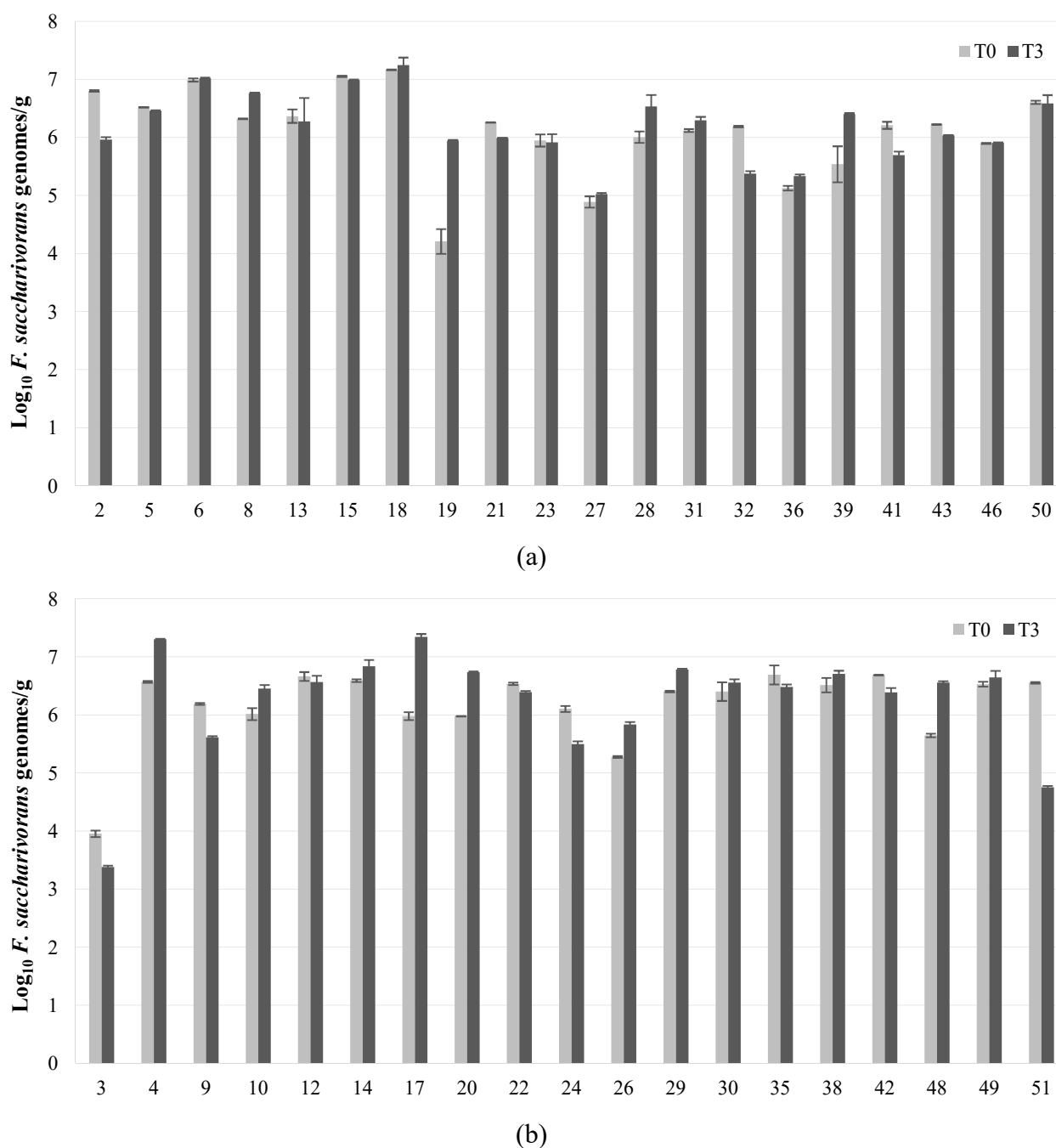


Figure 5.18. Quantification of *F. saccharivorans* in the treated (a) and control (b) groups of subjects, at the beginning (T0) and at the end (T3) of the intervention period.

Results obtained from the quantification of this species in the subjects that consumed the functional pasta (Figure 5.18a) showed a relevant increase only for subject 19, while no relevant decreases were observed. Regarding the analyses of the control group (Figure 5.18b), an increase in one logarithmic unit was observed for subject 17, while a decrease was observed for subject 51. On the overall groups, treated and control, no significant modulations of *F. saccharivorans* were observed (Figure 5.19). No differences were pointed out considering stratifications in overweight or obese subjects (Figure 5.20a, b; Figure 5.21), in the treated or control group, or from the intergroup comparisons.

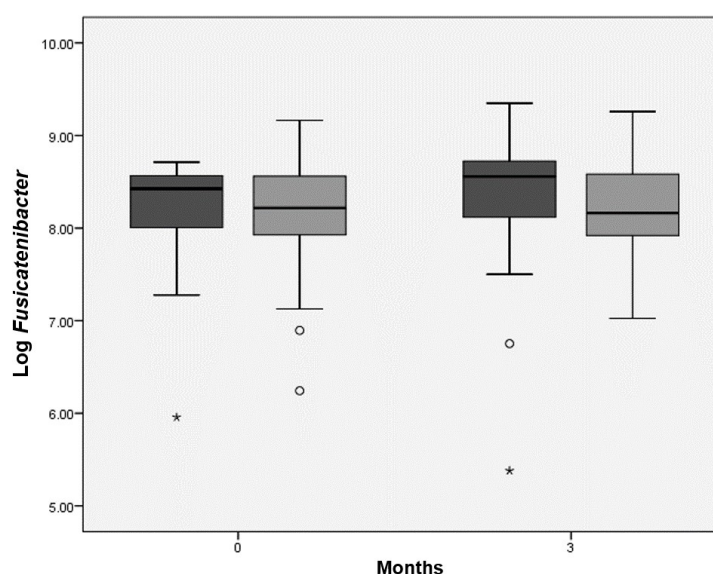
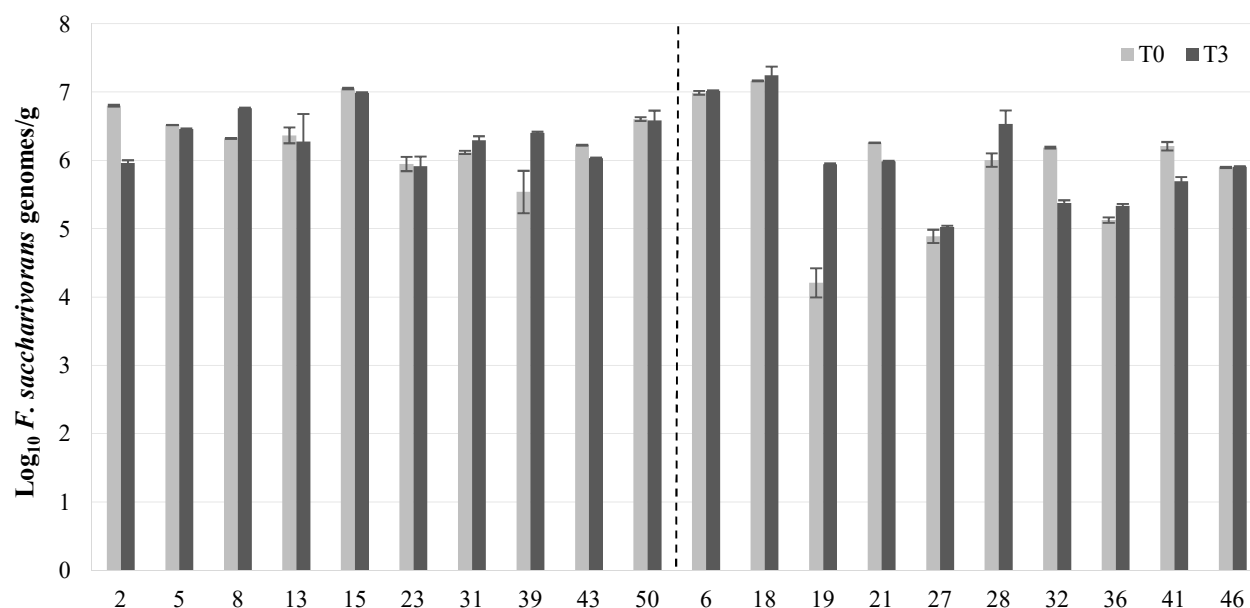
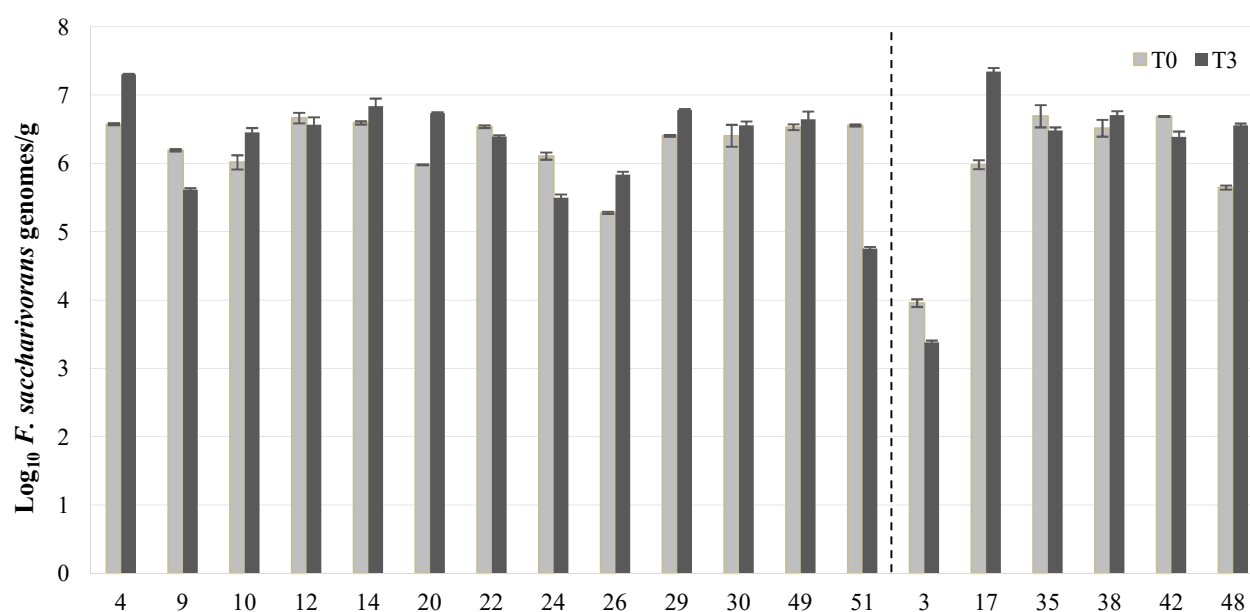


Figure 5.19. Box-plot representation of the amounts of *F. saccharivorans* at the beginning (month 0) and at the end (month 3) of the intervention period. Treated group is represented in light grey, control group is represented in dark grey.



(a)



(b)

Figure 5.20. Quantification of *F. saccharivorans* in the treated (a) and control (b) groups of subjects, at the beginning (T0) and at the end (T3) of the intervention period. Subjects are stratified according to their BMI, in overweight (left) and obese (right).

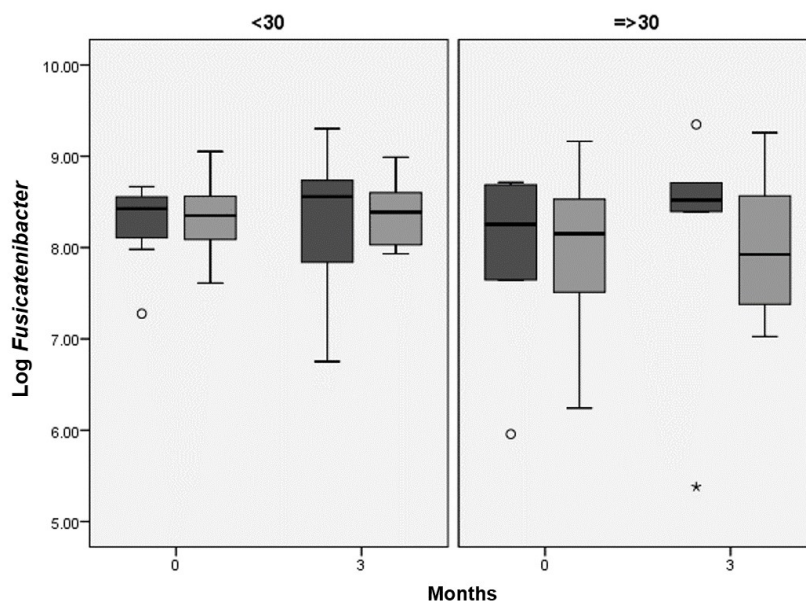


Figure 5.21. Box-plot representation of the amounts of *F. saccharivorans* at the beginning (month 0) and at the end (month 3) of the intervention period. Treated group is represented in light grey, control group is represented in dark grey. Subjects are stratified according to their BMI, in overweight (left) and obese (right).

So, results of the present study did not confirm previous findings. It should be considered that the evidence of the increase of this species after BC³⁰ ingestion derived from a single study carried out in an *in vitro* system, inoculated with the microbiota from a small number of subjects ($n = 2$). Thus, it is conceivable that extending the analysis to a larger number of subjects, observed effects can be different.

In general, the four microbial groups investigated did not resulted modulated following the intervention with the functional and/or control pasta. A similar result was observed in a study that compared a diet high in whole grain products with a diet with a lower content of these products. Also in this study no modulations were observed, for none of the analysed bacterial groups (Ampatzoglou *et al.*, 2015).

Variations of the analysed microbial *taxa* have been observed in specific subjects, but the variability of the individual response did not allow to identify significant differences in the whole groups, as reported elsewhere (Kovatcheva-Datchary *et al.*, 2015, Martínez *et al.*, 2013a). One of the reason could be attributed to the fact that the subjects of the clinical trial were not restricted to a specific diet; in fact, they were only asked to substitute the portion of pasta they usually had, with the functional or control pasta, keeping, for the rest, their own diets.

A strategy used in order to decrease the individual variability is the definition of subgroups of subjects with more homogeneous characteristics. In the present study, the stratification of individuals according to their BMI lead to a significant relationship between the intake of the functional pasta and the reduction of bifidobacteria in the overweight subjects. In addition, at the end of the trial, the amount of bifidobacteria found in the fecal samples of the obese subjects who consumed the functional pasta was significantly lower than the level found in samples of the obese subjects who consumed the control pasta, further confirming a non-bifidogenic effect of the functional pasta.

Since significant modulations of microbial *taxa* that were expected to be potentially responsive to the treatments were not observed, it is possible that the tested products had an effect on other microbial groups, or on certain species belonging to the investigated *genera*. In this perspective, the use of an open technique, such as a metagenomic approach, could provide a more complete view, and with a greater resolving power, about changes that may occur following a nutritional intervention. For example, investigation of other microbial groups involved in carbohydrates degradation, or of bacteria related to protein degradation would be interesting, considering the amounts of these components in the functional pasta (Chapter I).

Correlation with clinical data

In the contest of the clinical trial, a complete range of clinical parameters was monitored at the Department of Food and Drug, University of Parma. The most relevant results, observed after the consumption of the functional pasta, were related to positive effects about the modulation of specific metabolic and inflammatory markers (Angelino *et al.*, 2015).

A trend in decreasing the fasting glucose levels was observed, probably due to the presence of β -glucans (Cloetens, 2012). Regarding inflammatory markers, subjects who consumed the functional pasta exhibited significantly lower levels of C-reactive protein, comparing to subjects who ingest the control pasta, at the end of second and third month of the trial. Moreover, it was observed a significant decrease in PAI-1 levels (Plasminogen Activator Inhibitor-1) in the last four weeks, in subjects who consumed the functional pasta. Such anti-inflammatory effects may be imputable to both the β -glucans (Kristensen and Bügel, 2011), or the probiotic strain BC³⁰ (Mandel *et al.*, 2010).

The microbiological data obtained in the study were compared with the clinical results, and two interesting correlations emerged. The first, as reported previously, dealt with the decrease in the level of bifidobacteria following the treatment with the functional pasta in overweight subjects

(BMI <30 kg / m²). Since bifidobacteria are considered as positive bacteria in the gut (Arbolea *et al.*, 2016), their decrease may appear, at first, as a negative effect; however, a possible positive outcome related to this evidence can be speculated. The main products of bifidobacteria metabolism are acetate and lactate (De Vuyst *et al.*, 2014). Studies in murine models have investigated the possible role of acetate in adipogenesis (Hong *et al.*, 2005b) and, in particular, it has recently been proposed that an increase in acetate production could promote obesity (Perry *et al.*, 2016). In this perspective, a food able to lower *Bifidobacterium* levels and, consequently, the production of acetate, could be considered as beneficial for individuals in a overweight or obesity condition, and it will need further investigation.

Another interesting finding emerged when stratifying the subjects according to their blood glucose level, by setting the threshold of 100 mg/dL, defined by the International Diabetes Federation as a criterion for metabolic syndrome (Cloetens *et al.*, 2012). After the 3 months period of the trial, in individuals with glucose levels equal or above 100 mg/dL, subjects who consumed the functional pasta (in light grey) showed higher levels of *F. prausnitzii*, compared to subjects who consumed the control pasta (in dark grey) (Figure 5.22).

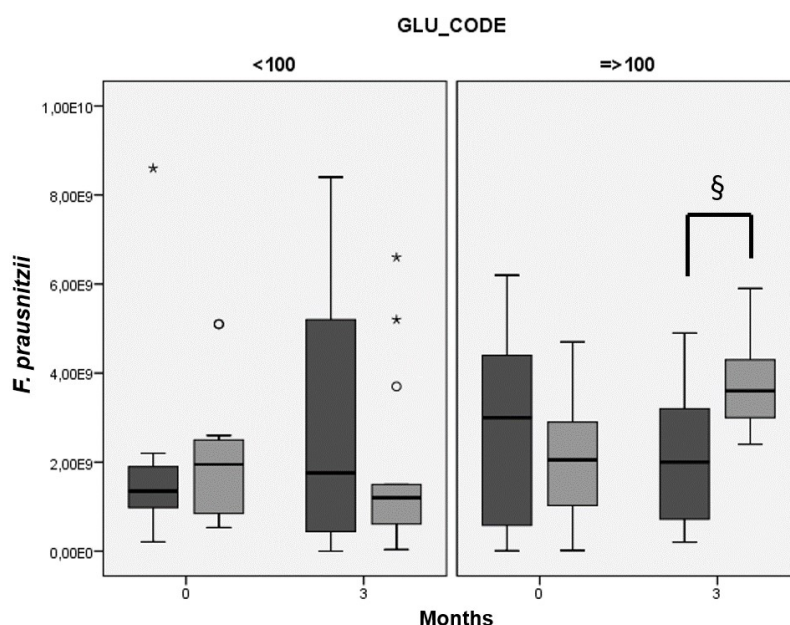


Figure 5.22. Box-plot representation of the amounts of *F. prausnitzii* at the beginning (month 0) and at the end (month 3) of the intervention period. Treated group is represented in light grey, control group is represented in dark grey. Subjects are stratified according to their glucose levels, in below 100 mg/dL (left) or equal or above 100 mg/dL (right).

F. prausnitzii is a positive microorganism in human gut microbiota, and it is able to influence host metabolism and health status, since it is a butyrate producer, and it presents immunomodulation properties (Miquel *et al.*, 2014). *F. prausnitzii* results decreased in various pathological conditions, and moreover, it is decreased in obese subjects compared to lean subjects (Walters *et al.*, 2014). Therefore, an increase in this species, which could positively enrich the intestinal microbiota, as well as acting on other clinical parameters, could certainly represent a positive outcome of a nutritional treatment.

As a final comment, with respect to clinical trials, a high inter-individual variability in response to a dietary intervention have been highlighted at both gut microbiota level and systemic level (Kovatcheva-Datchary *et al.*, 2015; Lampe *et al.*, 2013), that make not possible to define interventions that can be applied with the same expected output on large numbers of individuals, also considering the initial variability among individuals. For example, with regard to glucose metabolism, recent studies have emphasised how the glycemic response to a same nutritional intervention is individual-dependent (Kovatcheva-Datchary *et al.*, 2015; Zeevi *et al.*, 2015), and this variability is due to a combination of different factors, including the gut microbiota composition. The study of the characteristics of an individual, including microbiota, and their integration, constitutes the first step in the development of predictive systems about the response to a specific nutritional intervention (Lampe *et al.*, 2013). Such an approach will lead in the direction of the so-called “personalised nutrition”, aimed at ensuring, for example, controlled glucose levels in the blood, thus reducing the risk of developing diabetes and obesity (Zeevi *et al.*, 2015).

5.4. Concluding remarks

This study evaluated the effects of the consumption of a novel whole grain functional pasta, enriched in β -glucans and in the probiotic strain *B. coagulans* BC³⁰ on gut microbiota of overweight/obese subjects, in a 12-week parallel trial.

Overall modulations of total microbiota composition following ingestion of the functional product were investigated through PCR-DGGE analyses. Results showed that the functional pasta did not substantially modified microbiota profiles of the subjects. The different subjects were characterized by an inter-individual variability, at the beginning and at the end of the trial, and shared trends in terms of bands that characterised the profiles at the end of the trial were not observed. Similar results were obtained with PCR-DGGE on *Bifidobacterium* population.

Four bacterial *taxa*, that could be influenced by the different ingredients of the functional pasta, i.e. the genera *Bifidobacterium* and *Prevotella*, and the species *Faecalibacterium prausnitzii* and *Fusicatenibacter saccharivorans*, were specifically quantified through qPCR analyses, both in the treated and control groups of subjects. Results showed a marked inter-individual variability in the response to the consumption of the two food products. Considering the whole groups of subjects, the genus *Prevotella* was not significantly increased following the consumption of the two pasta, as well as the bifidogenic effect expected after assumption of whole grain fibre and β -glucans was not observed. Finally, *F. prausnitzii* and *F. saccharivorans*, that were expected to increase due to the probiotic strain, were not significantly modulated following the ingestion of the functional pasta. Only in restricted groups of subjects the expected modulations were observed. However, the stratification of the subjects based on their BMI resulted a successful approach to obtain more homogeneous subgroups of subjects, and it revealed a non-bifidogenic effect of the functional pasta, in overweight and obese subjects. A reduction of *Bifidobacterium* spp. could lead to a decreased production of acetate, and this could be considered a beneficial outcome for subjects presenting characteristics of overweight or obesity. Furthermore, an increase of *F. prausnitzii* was observed following treatment with the functional pasta in the subgroup of individuals with glucose levels exceeding 100 mg/dL, and it represented a positive effect of the consumption of the functional product.

In conclusion, even if investigated bacterial groups were not modified by the treatments in a significant manner, the correlation of microbiological and clinical data lead to some interesting findings. Extending the analysis to other microbial groups, e.g. through the use of a metagenomic approach, might reveal deeper modulation of the microbiota, in terms of composition and/or functions, which may help to clarify the results observed at the systemic level, on some clinical parameters.

This study was supported by the Italian Ministry for Development in the framework of the project “Pass-World - pasta e salute nel mondo - Industria 2015” [MI01_00138].

4. Conclusions

The area of functional foods has been receiving much attention in the last years, since foods are not intended any more as pure sources of nutrients and energy, but also as factors that can exert positive effects on the consumer, contributing to the maintenance of a healthy status. In this context, cereal based products present a range of beneficial features and, moreover, they can be used as vehicles of specific functional ingredients, for example probiotics and prebiotics. These functional components can directly act on gut microbiota, that has a deep impact on the overall health status.

The present thesis was developed in the context of an expansive research aimed at the creation of a novel whole grain functional pasta, enriched with a spore-forming probiotic strain, *Bacillus coagulans* GBI-30, 6086 (BC³⁰), and β -glucans from barley, that was performed in collaboration with many academic and industrial partners. In particular, the objectives of the present study have been the evaluation of the microbiological aspects related to the novel product and the assessment of its impact on gut microbiota.

In the first part of the dissertation, the production and the characterisation of the new functional food and of a respective control pasta has been shortly outlined, indicating that the novel pasta presented positive nutritional properties. The specific focus of this first part regarded the investigation of the stability of the probiotic strain, through culture-dependent and molecular analyses. On one hand, the technological properties of the probiotic strain during the pasta manufacturing process and preparation of the food have been investigated, showing that the probiotic strain survived the pasta production and cooking processes; in fact, *B. coagulans* BC³⁰ was found at high levels in the cooked product (above 10⁶ CFU/g), corresponding to a dose of approximately 10⁸ CFU for 100 g of ingested pasta. On the other hand, the probiotic strain was confirmed to resist to the transit in the gastrointestinal tract and to transiently colonise the gut compartment; in fact it was recovered in fecal samples at a concentration above 10⁶ CFU/g, during a 12-week trial in which the functional pasta was consumed daily. These findings expanded available knowledge on the stability of the probiotic strain *B. coagulans* BC³⁰, and specifically indicated that the functional pasta represented an effective vehicle for the probiotic strain.

In the second part of the thesis, dealing with Type 1 diabetes (T1D) context, a first case-control study was performed for assessing gut microbiota composition in children at risk for T1D

compared with healthy children. Even if a clear-cut differentiation between Cases and Controls was not observed, certain differences in composition were found through PCR-DGGE analyses, but not with 16S rRNA gene profiling, thus highlighting the impact of the use of different techniques in gut microbiota studies. With the first approach, three species, namely *Dialister invisus*, *Gemella sanguinis* and *Bifidobacterium longum*, were found to be associated to the pre-diabetic condition, together with an increase in gut permeability. Noteworthy, a species belonging to *Bifidobacterium* genus was found in the pre-pathologic condition, and another species, *Bifidobacterium adolescentis*, was found in pre-diabetic and diabetic microbiota at the baseline of the subsequent *in vitro* study. Thus, deepening the relevance of this microbial group for T1D development could be important, also considering the application of *Bifidobacterium* spp. in probiotic use.

An *in vitro* study was conducted with the TIM-2 model of the proximal colon, and it provided information about the effects (i.e. modulation of microbiota composition and short-chain fatty acid production) of the newly developed pasta and the functional ingredients on fecal microbiota from children with different susceptibility to T1D. In terms of composition, despite the variability in the response to a treatment observed in the different microbiota, specific diet-microbiota relationships were individuated, basing on the fact that a certain treatment produced certain same effects in the three microbiota. With this study new insights about the functional ingredients were obtained, e.g. the probiotic strain BC³⁰ induced the growth of specific bacterial groups when administered alone (e.g. increase in *Bifidobacterium* spp.) or in combination with other ingredients (e.g. increases in *Eubacterium* and *Lactobacillus* spp., when administered with β -glucans and concentrated starch, respectively). Regarding metabolite production, the functional pasta led to the highest butyrate yield in healthy and diabetic microbiota, that represented a positive output. However, since microbiota derived from a small number of children, this study represents a preliminary research, and further studies are needed also in the perspective of planning an intervention in children.

Finally, in the last part of the thesis, the effects of the consumption of the functional and control pasta were investigated in a 12-week clinical trial involving overweight/obese subjects. The consumption of the functional pasta did not substantially change the microbiota profiles of the subjects, as shown from PCR-DGGE analyses. Moreover, modulations of specific microbial groups reported to increase following administration of the different ingredients of the functional pasta were investigated through qPCR (*Prevotella* and *Bifidobacterium* spp., *Faecalibacterium prausnitzii* and *Fusicatenibacter saccharivorans*). Expected modulations were not observed in

the overall group of subjects, due to the higher inter-individual variability at the baseline and in the response to the treatment. Reducing this inter-individual variability by stratifying the population in smaller groups with more homogeneous characteristics resulted a successful strategy that unveil two interesting findings related to the consumption of the functional pasta, and obtained correlating microbiological and clinical data, i.e. a decrease in *Bifidobacterium* spp. in overweight subjects and an increase in *F. prausnitzii* in subjects with high glucose level.

In conclusion, the present research indicated that the novel developed pasta constitutes a high-quality product for its nutritional properties, for positive effects observed *in vitro* at the gut level, for certain beneficial evidences revealed from the clinical point of view, and from specific correlations between microbiological and clinical data. Further study would be helpful to deepen the knowledge of the effects of this new product, strengthen the integrated clinical-microbiological approach, and investigating subjects with homogeneous characteristics, in the future perspective of the so-called personalised diet.

Finally, the outline of this thesis could be proposed as a pipeline for the development of a new functional food, moving from the overall characterisation of the product, to the preliminary *in vitro* assessment of its effects at the gut microbiota level, to the integrated evaluation of its effectiveness in a clinical trial.

5. References

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6. List of publications

Different parts of this project have already been published or are in preparation for publication and have been presented as communications or posters in international conferences:

- results of Chapter 1 have been presented as a poster and published in a paper:
 - Martina A, Salvetti E, Felis GE, Torriani S. Introducing probiotics with the diet: the development of a new functional pasta enriched with *Bacillus coagulans*. Poster presented at the International Scientific Conference on Probiotics and Prebiotics - IPC2014, Budapest, Hungary; June, 24th-26th, 2014.
 - Fares C, Menga V, Martina A, Pellegrini N, Scazzina N, Torriani S. (2015). Nutritional profile and cooking quality of a new functional pasta naturally enriched in phenolic acids, added with β -glucan and *Bacillus coagulans* GBI-30, 6086. *J. Cereal Sci.* 65: 260-266.
- results of Chapter 3 have been presented as a poster and published in a paper:
 - Maffei C, Martina A, Corradi M, Quarella S, Nori N, Torriani S, Plebani M, Contreas G, Felis GE. Association between intestinal permeability and fecal microbiota composition in Italian children with beta cells autoimmunity at risk for type 1 diabetes. Poster presented at the 38th SOMED Congress of the Society for Microbial Ecology and Disease, Verona, Italy; October, 11th-13th, 2015.
 - Maffei C, Martina A, Corradi M, Quarella S, Nori N, Torriani S, Plebani M, Contreas G, Felis GE. (2016). Association between intestinal permeability and faecal microbiota composition in Italian children with beta cell autoimmunity at risk for type 1 diabetes. *Diabetes Metab. Res. Rev.* 32: 700-709.
- part of the results of Chapter 4 have been presented in an international conference:
 - Martina A. Investigation of microbial biomarkers in the gut microbiota of children at risk for type 1 diabetes. Presented at the 5th Beneficial Microbes Conference “Pre- and probiotics for lifelong human and animal health”, Amsterdam, The Netherlands; October, 10th-12th, 2016.

- part of the results of Chapter 2 and Chapter 5 has been presented as a poster:
 - Martina A, Felis GE, Angelino D, Zavaroni I, Vitaglione P, Fares C, Pellegrini N, Torriani S. Introducing probiotics and prebiotics with the diet: the development of a new whole grain functional pasta enriched with *Bacillus coagulans* and β -glucans”. Poster presented at Food Micro 2016, 25th International ICFMH Conference, Dublin, Ireland; July, 19th-22th, 2016.

Results of Chapter 2, Chapter 4 and Chapter 5 are in preparation for publication.

Other publications:

- Orrù L, Salvetti E, Cattivelli L, Lamontanara A, Michelotti V, Capozzi V, Spano G, Keller D, Cash H, Martina A, Torriani S, Felis GE. (2014). Draft genome sequence of *Bacillus coagulans* GBI-30, 6086, a widely used spore-forming probiotic strain. *Genome Announc.* 2: e01080-14.
- Salvetti E, Orrù L, Capozzi V, Martina A, Lamontanara A, Keller D, Cash H, Felis GE, Cattivelli L, Torriani S, Spano G. (2016). Integrate genome-based assessment of safety for probiotic strains: *Bacillus coagulans* GBI-30, 6086 as a case study. *Appl. Microbiol. Biotechnol.* 100: 4595-4605.